Regulation of Connexin43 by Phosphorylation in Heart Development

John C. Duncan

Follow this and additional works at: https://scholarsrepository.llu.edu/etd

Part of the Cardiovascular System Commons

Recommended Citation
Duncan, John C., "Regulation of Connexin43 by Phosphorylation in Heart Development" (1998). Loma Linda University Electronic Theses, Dissertations & Projects. 1033.
https://scholarsrepository.llu.edu/etd/1033

This Dissertation is brought to you for free and open access by TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. It has been accepted for inclusion in Loma Linda University Electronic Theses, Dissertations & Projects by an authorized administrator of TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. For more information, please contact scholarsrepository@llu.edu.
LOMA LINDA UNIVERSITY
Graduate School

REGULATION OF CONNEXIN43 BY
PHOSPHORYLATION IN HEART DEVELOPMENT

by

John C. Duncan

A Dissertation in Partial Fulfillment
of the Requirements for the Degree Doctor of
Philosophy in Anatomy

September 1998
Each person whose signature appears below certifies that this dissertation in his or her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

William H. Fletcher, Professor of Anatomy

David A. Hessinger, Professor of Physiology

Michael A. Kirby, Associate Professor of Anatomy

Cecilia W. Lo, Professor of Biology

Paul J. McMillan, Professor of Anatomy

Kerby C. Oberg, Assistant Professor of Anatomy
ACKNOWLEDGMENTS

I would like to express my appreciation to the individuals who helped me complete this study. I am grateful to Dr. William Fletcher for providing the facilities and support during my time at Loma Linda. I wish to thank Dr. Kerby Oberg and Dr. Cecilia Lo for their advice and the use of their facilities at different periods of time, and I wish to thank the other members of my guidance committee, Dr. Michael Kirby, Dr. Paul McMillan, and Dr. David Hessinger, for their advice and comments. I am grateful to Dr. Dale Laird for providing the CT360 for use in the western blotting. I am also thankful for laboratory assistance provided by Anna-Marie Martinez. I would also like to thank Tania Duncan, my wife, for her hours of proofreading and grammatical advice.

This work was supported by grants from the National Institutes of Health and the Veterans Administration, and by the Loma Linda University Department of Anatomy.
# TABLE OF CONTENTS

LIST OF TABLES .......................................................... vii  
LIST OF FIGURES ......................................................... viii 
ABBREVIATIONS ........................................................... ix 

CHAPTER ONE 

I. INTRODUCTION 

A. Cell-Cell Communication .................................................. 5  
   1. Connexin Proteins  
   2. Gap Junctions  

B. Growth Control .......................................................... 11  
   1. Gap Junction-Related Growth Arrest  
   2. Metabolic Cooperation  

C. Developmental Models ................................................... 15  
   1. Xenopus  
   2. Chick  
   3. Limb Development  

D. Early Mouse Development .............................................. 20  
   1. Compaction  
   2. Regulative and Mosaic Development  
   3. Egg Cylinder Stage  

E. Mouse Organ Formation ................................................ 29  
   1. Day Eight of Gestation  
   2. Embryonic Turning  
   3. Days Nine Through Sixteen  

F. Connexin Expression and Transgenics ............................... 43  
   1. Expression Patterns  
   2. Cx43 Knockout Mice  
   3. Visceroatrial Heterotaxia  
   4. Cx43 Overexpressors  

G. Regulation of Gap Junctional Communication .................... 48  
   1. Phosphorylation by Kinases  
   2. Protein Kinase C  
   3. Cyclic-AMP-Dependent Protein Kinase
### H. Goals and Objectives of Project

- CHAPTER TWO

### II. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>A. Timed Gestation</th>
<th>58</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Animal Housing</td>
<td>58</td>
</tr>
<tr>
<td>2. Animal Mating</td>
<td>58</td>
</tr>
<tr>
<td>3. Embryo Age Determination</td>
<td>58</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Tissue Collection</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Administration of Anesthetic</td>
<td>60</td>
</tr>
<tr>
<td>2. Dissection Procedures</td>
<td>60</td>
</tr>
<tr>
<td>3. Tissue Storage</td>
<td>60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Western Analysis</th>
<th>63</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gel Run Parameters</td>
<td>63</td>
</tr>
<tr>
<td>2. Tissue Preparation</td>
<td>63</td>
</tr>
<tr>
<td>3. Cytosolic and Membrane Separation</td>
<td>63</td>
</tr>
<tr>
<td>4. Protein Concentration Determination</td>
<td>63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>D. PKA Activity Assay</th>
<th>67</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tissue Preparation</td>
<td>67</td>
</tr>
<tr>
<td>2. Assay Parameters</td>
<td>67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>E. PKC Epsilon and PKC Delta Activity Assays</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tissue Preparation</td>
<td>70</td>
</tr>
<tr>
<td>2. Assay Parameters</td>
<td>70</td>
</tr>
<tr>
<td>3. PKC Purification</td>
<td>70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>F. Tissue Culture and Immunocytochemistry</th>
<th>74</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tissue Digestion</td>
<td>74</td>
</tr>
<tr>
<td>2. Cell Plating</td>
<td>74</td>
</tr>
<tr>
<td>3. Immunohistochemical Procedures</td>
<td>74</td>
</tr>
</tbody>
</table>
CHAPTER THREE

III. RESULTS

A. Connexin Protein Analysis

B. Cyclic-AMP-Dependent Protein Kinase
   1. PKA Protein Analysis
   2. Activity Assay Results

C. Novel Protein Kinase C Family Members
   1. PKC Delta Expression
   2. PKC Epsilon Expression
   3. Combined Assay Results

D. Heart Tube to Loop Fusion (8.5-11.5 dpc)
   1. Cx43 Expression
   2. PKA Expression and Activity
   3. PKC Delta and Epsilon Expression and Activity

E. Chamber Formation and Growth (12.5-15.5 dpc)
   1. Cx43 Expression
   2. PKA Expression and Activity
   3. PKC Delta and Epsilon Expression and Activity

F. Late Gestation, Birth and Adulthood (16.5-30.5, 76 dpc)
   1. Cx43 Expression
   2. PKA Expression and Activity
   3. PKC Delta and Epsilon Expression and Activity

CHAPTER FOUR

IV. DISCUSSION AND CONCLUSIONS

A. Connexin43 Regulation

B. Transgenics

C. Protein Kinases

D. Summary

V. APPENDIX

VI. REFERENCES
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tissue Usage for Western Analysis</td>
<td>65</td>
</tr>
<tr>
<td>2. Tissue Usage for Kinase Activity Assays</td>
<td>69</td>
</tr>
<tr>
<td>3. Protein Kinase A Activity Assay Results</td>
<td>88</td>
</tr>
<tr>
<td>4. Combined Protein Kinase C Isozyme Activity Assay Results</td>
<td>115</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Suggested Topological Structure of Connexin Proteins</td>
<td>6</td>
</tr>
<tr>
<td>2. Cartooned Gap Junction</td>
<td>8</td>
</tr>
<tr>
<td>3. Cartooned Intercellular Communication Via Gap Junctions</td>
<td>9</td>
</tr>
<tr>
<td>4. Embryo at Egg Cylinder Stage</td>
<td>27</td>
</tr>
<tr>
<td>5. Embryo at Gestational Age of Eight Days</td>
<td>30</td>
</tr>
<tr>
<td>6. Embryo at Gestational Age of Eight and One-Half Days</td>
<td>33</td>
</tr>
<tr>
<td>7. Cartooned Embryonic Turning</td>
<td>34</td>
</tr>
<tr>
<td>8. Embryo at Gestational Age of Nine and One-Half Days</td>
<td>37</td>
</tr>
<tr>
<td>9. Embryo at Gestational Age of Ten and One-Half Days</td>
<td>38</td>
</tr>
<tr>
<td>10. Embryo at Gestational Age of Eleven and One-Half Days</td>
<td>40</td>
</tr>
<tr>
<td>11. Hearts of Gestational Age Twelve through Sixteen Days</td>
<td>42</td>
</tr>
<tr>
<td>12. Western Analysis of CX43</td>
<td>80</td>
</tr>
<tr>
<td>13. Western Analysis of PKA</td>
<td>83</td>
</tr>
<tr>
<td>14. Graph of PKA Activity</td>
<td>86</td>
</tr>
<tr>
<td>15. Western Analysis of PKCδ</td>
<td>89</td>
</tr>
<tr>
<td>16. Western Analysis of PKCδ continued</td>
<td>90</td>
</tr>
<tr>
<td>17. Western Analysis of PKCe</td>
<td>91</td>
</tr>
<tr>
<td>18. Western Analysis of PKCe continued</td>
<td>92</td>
</tr>
<tr>
<td>19. Graph of Combined PKCδ and PKCe Activity</td>
<td>105</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>AER</td>
<td>Apical ectodermal ridge</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C subfamily, calcium insensitive, phospholipid-dependent</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Ionic calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3', 5'-monophosphate</td>
</tr>
<tr>
<td>cc</td>
<td>Cubic centimeter volume</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine 3', 5'-monophosphate</td>
</tr>
<tr>
<td>CKI</td>
<td>Protein kinase C inhibitor protein</td>
</tr>
<tr>
<td>cPKC</td>
<td>Conventional protein kinase C subfamily, calcium dependent</td>
</tr>
<tr>
<td>Cx “#”</td>
<td>Connexin followed by the molecular weight of the protein, i.e. Cx43 is equivalent to connexin43</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIFP</td>
<td>Diisopropyl fluorophosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
</tbody>
</table>
EGTA  Ethyleneglycol- bis (β-aminoethyl ether) - N, N'- tetraacetic acid
ELISA  Enzyme-linked immunosorbent assay
FBS    Fetal bovine serum
FITC   Fluorescein isothiocyanate
IP₃    Inositol 1,4,5-trisphosphate
K+     Ionic potassium
MES    2-([N-morpholino]ethanesulfonic acid
mRNA   Messenger ribonucleic acid
no.kD  Kilodalton, measure of protein weight
no.dpc Days post-conception
no. dpp Days post-partem
nPKC   Novel protein kinase C subfamily, calcium independent, DAG dependent
PBS    Phosphate-buffered saline
PKA    Cyclic-AMP-dependent protein kinase (protein kinase A)
PKC    Calcium-sensitive, phospholipid-dependent protein kinase
PKCα   Protein kinase C alpha isoform
PKCβ   Protein kinase C beta isoform
PKCδ   Protein kinase C delta isoform
PKCε   Protein kinase C epsilon isoform
PKCθ   Protein kinase C theta isoform
PKCi   Protein kinase C iota isoform
PKC\(\lambda\)  Protein kinase C lambda isoform
PKC\(\mu\)  Protein kinase C mu isoform
PKC\(\eta\)  Protein kinase C nu isoform
PKC\(\zeta\)  Protein kinase C zed isoform
PKI  Protein kinase A inhibitor protein
PMSF  Phenylmethylsulfonyl fluoride
RIPA  Tissue solubilization buffer
RNA  Ribonucleic acid
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS  Tris-buffered saline
TPBS  0.05% tween in PBS
TTBS  Tween tris-buffered saline
VAH  Visceroatrial heterotaxia
ABSTRACT

REGULATION OF CONNEXIN43 BY PHOSPHORYLATION IN HEART DEVELOPMENT

by

John C. Duncan

Serine/threonine protein kinases have been shown to reversibly regulate cell-cell communication through gap junctions formed by connexin 43 (Cx43) (Godwin et al., 1993). The Cx43 protein expression levels are unclear and what role phosphorylation plays in the regulation of signal transduction through these gap junctions during heart formation. Reportedly, mutations which replace potentially phosphorylatable serines within the Cx43 protein are associated with heart malformations (Britz-Cunningham et al., 1995), highlighting the importance of determining the levels of Cx43 and the activities of PKA and PKC during heart development.

Western analysis performed on hearts from developmentally significant time points revealed principally high molecular weight forms of Cx43. Metabolic labeling procedures confirmed that these forms of Cx43 were phosphorylated, suggesting that the development of a functional, four-chambered heart requires gap junctional communication provided by Cx43 during embryogenesis which is differentially regulated by phosphorylation.

PKA expression levels were relatively constant over the developmental time frame except for an increase at 10.5 dpc and minor fluctuations during the completion of heart
chamber formation. Thereafter, a steady increase in the amount of enzyme was noted in all time points examined through adulthood.

PKCε was detected below adult concentrations at all time points at levels that were relatively constant except for increases at 10.5 and 15.5 dpc. Protein quantity increased steadily from 16.5 dpc to adulthood, starting from a lower level than that detected at 15.5 dpc and rising to adult concentrations.

PKCδ had the most variable expression of the protein kinases examined, with rapid fluctuations occurring during heart development. Again, detected levels peaked at 15.5 dpc and increasing protein quantities mimicked the rise in PKCε to adult levels starting at 16.5 dpc, except that at birth protein expression declined.

To further understand the importance of these varying levels of expression during organogenesis, in vitro phosphorylation assays for determination of protein kinase activity were completed. PKA activity peaked at days corresponding to the completion of heart-tube looping, chamber formation and preparation for birth. Combined nPKC activity levels were relatively high in the early developmental period; thereafter activity continually decreased.
CHAPTER ONE
INTRODUCTION

Out of a group of 29 congenital malformations found to be occurring with increasing frequency in the past few years, half were cardiac defects (Edmonds and James, 1990). Increasing diagnoses of cardiac defect rates are acknowledged to be partially due to improvements in health care and advances in diagnostic techniques. Progress in medical science has resulted in the diagnosis of more mild or asymptomatic defects as well as more serious defects that until recent years would not have been discovered because the children would not have survived. The resulting increase in the numbers of children with cardiac defects accentuates the need for information in preventing these birth defects and treating this new patient population.

In contemplating a solution to the massive problem of infant death relating to birth defects, the Center for Disease Control (CDC) stated, "Epidemiologic and basic research are integral to the development of prevention programs for infant mortality," (CDC, 1989) underscoring the importance of basic research related to this important health issue. Further, just one year after the CDC's statement, Lynberg and Khoury (1990) reported that according to a survey of recorded causes of death, the leading threats to infant survival in the United States were cardiovascular system and central nervous system birth defects. Not only is pain involved in the loss of a long-expected child and a cost in human life, but health care costs for therapy and care of children with birth defects exceed one billion dollars annually. Thus, the problem of birth defects is not diminishing and will not
begin to attenuate until serious attention is given to developmental issues on the part of
basic science researchers.

For many years the size of the mammalian embryo was thought to prohibit in-depth
study of early developmental stages; techniques for handling embryos were inadequate.
However, in the past twenty years this trend has changed. Advances in technology have
made it possible to do more extensive studies on the mammalian embryo. With the ever-
expanding study of this stage of life has come a wealth of information about what steps
development takes. However, what we comprehend about this process is far outweighed
by those things that continue to elude our understanding.

In addition to the fact that defects of the cardiac system are reported to be the most
common birth defects, there is evidence that mutations or changes in the gene coding for
the connexin43 (Cx43) protein may contribute to this increasing trend (Britz-Cunningham
et al., 1995; Reaume et al., 1994). Intercellular communication via gap junctions is
thought to play a role in many developmental events including construction of a
functional, four-chambered heart (Gros et al., 1979). Cell-cell exchange of signal
molecules like cAMP, inositol 1,4,5-trisphosphate (IP3), Ca^{2+}, and probably other species,
is thought to be made possible by Cx43 gap junctions (Murray and Fletcher, 1984) which
unite cardiac cells (Beyer, 1993). Passage of these molecules from the cytoplasm of one
cell to the cytoplasm of another, which may be responsible for directing formation of the
heart, is at least partly regulated by phosphorylation and dephosphorylation events (Saez
et al., 1986; Godwin et al., 1993). Transfer of cyclic nucleotides presumably results in
activation of protein kinases and phosphorylation of component connexins (Musil et al., 1990b). Given this, it is important to evaluate amounts and activities of involved protein kinases and Cx43 during heart development. This study observes regulation of Cx43, a protein thought to play a significant developmental role not only in the heart but many other systems in the embryo.

A. Cell-Cell Communication

1. Connexin Proteins

Cx43 is one of the gap junction proteins called connexins, encoded by a multigene family (Haeflinger et al., 1992; Bennett et al., 1991; Fishman et al., 1991a; Beyer et al., 1990; Willecke et al., 1990). Seventeen cDNAs representing coding sequences of connexins have been characterized and are listed in GenBank. These connexins have been named for their molecular weight in kilodaltons (kD) deduced from these cDNAs (Revel et al., 1992; Paul et al., 1991). Topological structure of the connexin protein family is accepted to be composed of four membrane-spanning domains, two extracellular loops, one intracellular loop and cytoplasmic amino (N) and carboxyl (C) -termini established on analysis done by many investigators (Goodenough et al., 1988; Hertzberg et al., 1988; Milks et al., 1988; Zhang and Nicholson, 1994) (Fig. 1). Amino acid sequence homology is greatest among connexin proteins in the four membrane-spanning domains and in the extracellular loops, while the cytoplasmic loop and C-terminus of the proteins exhibit the greatest diversity. Because of marked diversity localized in these areas, the cytoplasmic
Figure 1. Structural analysis of the topological structure of connexin proteins suggests there are four membrane-spanning domains, numbered here 1 to 4. Domain 3 is thought to line the channel of the connexon. Two extracellular loops contain conserved cysteines, noted here as C. The intracellular loop is denoted as CL; the cytoplasmic N- and C-termini along with the amino acid sequence thought to regulate channel state by phosphorylation are recognized by their biochemical notations. Modified from Stagg and Fletcher (1990).
loop and carboxyl tail are thought to be responsible for unique functions or regulation of channels formed by different connexins (Reaume et al., 1995).

Within extracellular loops there are characteristically spaced, conserved cysteines. As reported by John et al. (1991), at least one pair of these cysteines hold the loop between transmembrane segments 1 and 2 to the corresponding loop between transmembrane segments 3 and 4 by formation of an intramolecular disulfide bond. The other two pairs of cysteines are thought to form similar intramolecular disulfide bonds in the oxidizing environment of the extracellular space. The third transmembrane domain is believed to line the wall of the hexameric hemichannel (a connexon) in the cell membrane (Fig. 2) formed by oligomerization of six of these proteins (Makowski et al., 1977; Nishi et al., 1991; Hall and Gourdie, 1995).

When a connexon is aligned with a like hemichannel in an opposing cell membrane, a hydrophilic passage is formed between the cytoplasms of the two cells (Fig. 3). Intercellular communication is then possible, involving low molecular-weight (<1000 Da) metabolites, ions, and signal transduction molecules such as cyclic nucleotides (Murray and Fletcher, 1984), inositol 1,4,5-trisphosphate (IP3), and Ca^{2+} (Oyamada et al., 1994; Berthoud et al., 1992). Direct passage of these molecules between cytoplasms of adjoining cells is regulated by the sensitivity of connexins to voltage (Spray et al., 1979), cyclic nucleotides (De Mello, 1988; Saez et al., 1986), changes in pH (Turin and Warner, 1977, 1980), phosphorylation (Godwin et al., 1993; Musil et al., 1990b), and pharmacological agents (Goliger and Paul, 1994). This communication can also be
Figure 2. A gap junction cartooned here is constructed from membrane bound proteins called connexins which oligomerize as a heximeric hemichannel, or connexon, in the cell membrane (Makowski, et al., 1977). The diameter of a connexon is thought to be about six nm. Center-to-center distance between two connexons within a gap junction is believed to be on the order of nine nm.
Figure 3. This representation courtesy of William H. Fletcher, Ph.D. depicts intercellular communication by a connexon that is aligned with a like hemichannel within the adhered cell membranes. A hydrophilic passage is formed between the cytoplasms of two cells. It would take many such structures to form a gap junction. Direct cytoplasmic intercellular communication involving cAMP, inositol 1,4,5-trisphosphate (IP₃), Ca²⁺, and a host of other molecules is then possible. Metabolites are shown experimentally to be just one of the substances capable of this type of transport, giving cells the ability for metabolic cooperation.
regulated by alignment of two homomeric hemicannels where the six identical connexins of each are different, in which case the channels are referred to as heterologous or heterotypic (Brink et al., 1997).

2. Gap Junctions

Assemblies of these membrane channels form "plaques" called gap junctions that form the infrastructure of intercellular communication within neighboring cell membranes. Gap junctional plaques in the adult mammalian myocardium can occupy surface areas on the cell membrane that are 2-µm in diameter and contain thousands of channels arranged as clusters with intervening spaces (Chen et al., 1989), or as single aggregations 1-µm in diameter without any intervening space (Green and Severs, 1984; Green et al., 1993).

Gap junctions show a change in their geometrical structure and organization during development. The first arrangement is that of a linear row. The only arrangement reported by Mazet (1977) to be found in *Xenopus* (tree frog), these linear gap junctions have also been found in the developing mammal but seem to be restricted to the nodal region of the adult heart (Gros et al., 1978; Larsen, 1977; Sugi and Hirakow, 1986). The second geometry present in the embryonic and non-mammalian adult heart is that of a circle, or associated and anastomosing circular complexes (Skepper and Navaratnam, 1986; Shibata et al., 1980; Mazet and Cartaud, 1976; Sibata and Yamamoto, 1979). Again, gap junctions composed of a circular complex if noted in the adult mammalian heart are found in the nodal region. Gap junctions in the adult working myocardium are composed of single aggregations or large clusters, and although these types of gap
junctions have been reported in developing systems they are believed to be the adult forms (Baldwin, 1979; Severs, 1990; Masson-Povet et al., 1979). The different geometrical arrangements themselves may contribute attributes related to the speed with which signals are transmitted through the junction, thus relating to regulation of intercellular communication provided within a tissue (Hall and Gourdie, 1995).

Intercellular communication via gap junctions is thought to play a role in many cellular activities; hypotheses for use of this transmission of signals have arisen from discovery of their presence in almost every cell type. One such cellular activity is control of cell division. Most of this information has been applied to cancer (Eghbali et al., 1991; Zhu et al., 1991) where restraint over growth has been eliminated in a subset of cells. However, embryogenesis is also a state of rapid growth where intercellular communication may be used to strictly maintain control throughout development.

B. Growth Control

A phenomenon discovered to support the idea that gap junctions might play a role in growth arrest was reported by Stoker (1964), who observed that uncontrolled growth of certain transformed cells was inhibited when they were grown in contact with normal cells (Stoker et al., 1966; Borek and Sachs, 1966; Eagle et al., 1968). Further studies reported that cell interaction was directly responsible for this inhibition and there was no evidence to support the notion that a factor in the extracellular medium was involved (Stoker, 1967; Bertram, 1979; Bertram and Faletto, 1985).
1. Gap Junction Related Growth Arrest

Loewenstein (1979) proposed that growth-controlling signal flow consisted of five stages: generation of the signal, transmission, reception, comparison of signals, and effector processes of cell division; further, cell-cell links in the transmission line were gap junction membrane channels. This concept was not to imply that extracellular signals have no role in growth regulation but was only to be applied to areas where spatial resolution was needed. Thus, ample room is left for actions of hormones, nutrients, and growth factors.

Evidence to support this line of reasoning was gathered by the use of two experimental designs. In one, reported cyclic-AMP-dependent mechanism of phosphorylation was exploited to experimentally raise the level of communication between transformed and normal cell types (Azarnia et al., 1981; Flagg-Newton and Loewenstein, 1981; Flagg-Newton et al., 1981; Radu et al., 1982; Wiener and Loewenstein, 1983; Loewenstein, 1985). Treatment of cocultures with forskolin or with phosphodiesterase inhibitors, RO 20-1724 or isobutyl methyl xanthine, resulted in an increased frequency of communication and a decreased growth rate of transformed cells. The second experimental design developed by Pitts et al. (1981) utilized retinol or retinoic acid to block communication between transformed and normal cell types in coculture. Abolition of intercellular communication resulted in loss of growth control. Mehta et al. (1986) further reported that effects on growth of cells treated with forskolin or phosphodiesterase
inhibitors in conjunction with retinoids resulted in inhibition of junctional communication by retinoids superseding the actions of forskolin or phosphodiesterase inhibitors.

In the growth-control system proposed by Loewenstein (1979) and supported by experimental evidence cited above, cell growth is controlled simply by chemical concentration of intracellular molecular signals. Thus, as the number of cells increases intracellular volume increases proportionally resulting in signal dilution. Accordingly, division ceases when the signals to divide are sufficiently dilute within the cell volume. Component cells of a tissue receive these signals from several sources: extracellular matrix or fluids, contacting neighboring cells, and from within cells themselves. Loewenstein’s proposal also states that once equilibrium is achieved, growth and size of the organ or organism is set. With this, a new role for intercellular communication arises: maintaining tissue homeostasis.

2. Metabolic Cooperation

Subak-Sharpe et al. (1966, 1969) provided the first evidence for metabolic cooperation by using a mutant strain of hamster fibroblast cells, BHK21, that lacked inosine pyrophosphorylase activity (IPP-) which rendered the cells incapable of incorporating [3H] hypoxanthine into DNA. Only when gap junctional cell contact between BHK (IPP+) and BHK21 (IPP-) was made in coculture are IPP- cells able to incorporate tritium radiolabeled hypoxanthine. Green (1992) further reported that cells resistant to culture in lethal nucleotide analogues died when cocultured with normal cells
that were able to incorporate these lethal agents. The mutant cells received this "kiss of death" only when they established cell-cell communication with these nucleotide-incorporating cells. From these results it was clearly important to examine other systems in a living organism where tissues would benefit from metabolic cooperation maintained by gap junctional communication.

Sustaining metabolic homeostasis would be difficult in an avascular setting such as the lens; thus, gap junctional communication is thought to play a critical role in maintenance of this tissue (Mathias and Rae, 1985; Goodenough et al., 1980). Before formation of the vascular system in the embryo, the level of participation of cellular communication in dispersal of metabolites is a possible mechanism by which some compounds could be delivered to cells distant from the nearest vascular supply. Also, it has been hypothesized that astrocytes, in their assigned property of buffering K⁺, would increase their buffering capacity by passing these ions away from the active neurons through membrane channels to other astrocytes, thus effectively increasing the volume of the buffer sink and increasing the efficiency of astrocytes to maintain homeostasis in neural tissue (Trachtenberg and Pollen, 1970).

Given the results discussed above it seems likely that if metabolites and other molecules can pass from cell to cell through gap junctional channels, then perhaps molecules that direct cell differentiation, like cellular morphogens, could also be passed during development. At a predetermined point, however, these chemicals would have to be restricted in their movement. Pitts et al. (1988) applied this concept to the skin, a
system that creates and maintains a uniform epidermal thickness which is believed to require a tight regulation of keratinocyte growth and differentiation. Goliger and Paul (1994) reported that in rodent epidermal layers there are clearly distinct expression patterns for Cx26, Cx31.1, Cx37, and Cx43 which would be expected in a system that would be transporting morphogens and requiring discrete communication. Further, it appears that multiple gap junction gene expression (Cx43, Cx32) contributes to epidermal and follicular morphogenesis, and differential expression of these gap junction genes coincides with epidermal differentiation and maturation (Risek et al., 1992). Support for the theory that transport of morphogens and tissue differentiation is related to regulated gap junction expression continues to grow, not just in rat epidermal tissue but in the following embryo models as well.

C. Developmental Models

1. Xenopus

One of the first models used to explore the importance of gap junctions in vertebrate development was the Xenopus embryo (Guthrie, 1984). This was a direct result of renewed interest in developmental mechanisms which grew from information on the ability of the connexin protein to form channels that could transmit information about growth, participate in metabolic cooperation, and transport cellular morphogens. Reasons for working with Xenopus as a laboratory model were that eggs of this animal are easily manipulated and ultimate fates of each cell of an 8- to 16-cell stage embryo are known (Guthrie et al., 1988).
It has long been recognized that cells of the early embryo can communicate with one another. Many early studies of gap junctional communication used blastomeres of newt or *Xenopus* embryos. Injecting dyes with differing molecular weights, Guthrie (1984) demonstrated gap junctional communication in the 16-cell stage *Xenopus* embryo. Retention of larger molecular weight dyes but transfer of lower molecular weight dyes led her to conclude that communication through gap junctions began at the 16-cell stage. Transfer of dye among cells of the *Xenopus* embryo before this stage of development is cell-cycle dependent and is most likely due to cytoplasmic bridges as illustrated by visualization of FITC-dextran (Mr 5000) movement into sister cells (Su et al., 1990; Cardellini et al., 1988). Dye transfer analysis in the *Xenopus* embryo is modified over the developmental period as the embryo matures from the 16- to 64-cell stage. During this period, communication is being modulated until some areas of the 64-cell stage embryo only receive dye while others only donate dye. However, during this sequence of events dye is never transferred across the primary cleavage which establishes the dorsal/ventral axis of the embryo (Guthrie et al., 1988).

Gap junctions were shown to be present in pigment epithelial cells of *Xenopus* retina (Dixon et al., 1974) and were correlated with the non-neural stimulus-response system in *Xenopus* epithelium (Chuang-Tseng et al., 1982). Despite widespread support for intercellular communication being critical in secondary cellular interactions (Grunz, 1985), there was very little evidence to identify precise roles played by gap junctions in embryonic
development. However, this was to change with the generation of antibodies against isolated connexin proteins found in rat liver gap junctions.

Warner and colleagues (1984) raised two polyclonal antibodies to the 27 kD molecular weight protein in rat liver gap junctions. A third antibody was raised against an extracellular matrix glycoprotein as a control. When injected into a single cell on the dorsal side of the 8-cell stage *Xenopus* embryo, gap junction antibodies resulted in the shut down of gap junction communication in later cell divisions as measured by cell-cell fluorescent dye transfer. The control antibody showed no such effect, confirming it was interaction of the antibody with the connexins that blocked communication. When allowed to develop until the early tadpole stage, these animals had specific central nervous system abnormalities and in some cases the entire eye and parts of the central nervous system on the injected side were completely absent. These findings provided evidence that when junctional communication was disrupted, development could not progress in the normal fashion (Warner, 1985).

2. Chick

The chick embryo has also provided an advantageous model for examining molecular exchanges facilitated by gap junctions. Several investigators have reported an increase and then decline in levels of gap junctional plaques in relation to differentiation of ocular tissues (Fujisawa et al., 1976; Sheffield, 1980). In chick liver development, decreases in gap junctions occur concurrently with overt differentiation after embryonic stage 28 (6 days of incubation). This parallels a decline in growth of liver tissue as well,
possibly reflecting a mechanism governing early expansive growth and then decaying with subsidence of this mitotic activity (Meyer and Overton, 1983). The phenomenon of gap junctions disappearing (Eley and Shelton, 1976) or decreasing in size or frequency with the maturation of an organ is not isolated to chick development but also occurs during the maturation of rat seminiferous tubules (Meyer et al., 1977). These studies support the hypothesis that in developing organs gap junctional communication could play important roles in organization and differentiation of cellular constituents as predicted by the “signal dilution” hypothesis (Loewenstein, 1979) discussed previously.

3. Limb Development

Further evidence that connexin proteins have an important place in tissue differentiation is observed in limb development. Two sources of mesenchyme contribute to limb formation: mesenchymal cells from somites giving rise to musculature, and lateral plate mesenchyme giving rise to skeletal framework (Chevallier et al., 1977; Christ et al., 1977; Kieny and Chevallier, 1979). The apical ectodermal ridge (AER) forms within the overlying ectoderm due to biochemical signals (morphogens) emanating from the lateral plate mesenchyme. Evidence for this has been gathered by transplantation of these mesenchymal cells to other locations on the embryo and the resultant formation of limb buds irrespective of the origin of the ectoderm (Harrison, 1918). The AER then reciprocates with another set of signals that act on the underlying mesenchymal cells to promote their growth as well as maintain this tissue in an undifferentiated state and,
directly or indirectly, provide positional information along the proximal-distal axis (Kosher et al., 1979).

Closer examination of expression patterns of gap junctions in the limb by in-situ hybridization revealed a high concentration of Cx43 transcript in the AER and posterior sub-ridge mesoderm with a contrasting low concentration of Cx43 RNA elsewhere (Dealy et al., 1994). Expression of this connexin transcript seems to have produced a greater concentration of Cx43 gap junctions in the posterior polarizing region of the limb, showing sensitivity to fibroblast growth factor 4 (FGF4); this suggests that growth factor signaling and gap junctional communication are coordinately regulated (Makarenkova et al., 1997). These results suggest that activities of cells within the AER and sub-ridge mesoderm are integrated as reflected in the outgrowth of this tissue in response to signals from the overlying ectoderm, but these cellular components are compartmentalized from surrounding tissues (Dealy et al., 1994).

Interruption of intercellular communication provided by this concentration of Cx43 junctions in the ectodermal structure causes developing limbs to be truncated as shown by implantation of beads soaked in 1 mg/ml retinoic acid (Tickle et al., 1989). Further, Green et al. (1994) reported that apical ectodermal ridges treated with retinoic acid caused abolition of labeling by antipeptide antibodies specific to Cx43 gap junction protein of both epithelial and mesenchymal tissues. As a result of this treatment the ridges underwent flattening and buds were rimmed by non-ridge ectoderm which had no
detectable expression of Cx43. All this implies that differentiation of the AER tissues and proper growth of limbs is dependent on gap junctional communication involving Cx43.

These experimental data would have been more conclusive had another method been used to reduce gap junctional communication; retinoic acid was once thought to be a morphogen in the limb. The use of beads soaked in retinoic acid may have resulted in disruption of the natural gradient of this compound in the developing limb that contributed to truncation of the limb structures, independent of reported effects on Cx43 protein expression (Green et al., 1994). Irrespective of what other morphogenic effects retinoic acid may have had, loss of labeling by antipeptide antibodies is significant. This suggests that perhaps the morphogenic properties of this compound result from its effect on Cx43 expression and thus its possible regulatory role in cell-cell communication. Integration of the role of retinoic acid with those of FGF4, sonic hedgehog (Shh) (Laufer et al., 1994; Niswander et al., 1994) and many other factors that direct cellular growth and differentiation during limb formation remains to be elucidated.

D. Early Mouse Development

In the context of cellular differentiation and mammalian development, the activities of gap junctions are frequently probed for clues. Mice expressing transgenes are becoming the developmental models of choice because of their short generation time and the numbers of embryos that can be obtained. By studying these animal models to gain an
understanding of how signals for growth and tissue organization are transmitted and interpreted during embryogenesis, we gain insights into factors that guide normal development. Insertion of connexin proteins into cell membranes, and operation, regulation and control of gap junctional communication have become the focus of much research in recent years.

1. Compaction

Messenger RNA for Cx43 first appears at the preimplantation, 4-cell stage of the mouse embryo, but protein and gap junctional communication, assayed by dye transfer, are not present until the 8-cell stage (Lo and Gilula, 1979a). At this time the cells, or blastomeres, polarize and flatten on each other, a process known as compaction. Compaction of the mammalian zygote precedes the establishment of the trophoblast and inner cell mass lineages. The morula (8-cell stage embryo) thus is outlined by a smooth surface and boundaries of individual blastomeres are no longer easily distinguished. Formation of tight junctions at this time results in a permeability seal that makes possible accumulation of fluid and formation of a blastocyst, which consists of a single layer of trophoblast cells forming the shell of a hollow sphere. Internally, at one pole of this sphere, is an aggregation of smaller cells defined as the inner cell mass which will give rise to the embryo proper. The temporal alignment of the formation of gap junctions with the beginning of compaction appears to be a vital step of development.

Evidence supporting the notion that gap junctional communication plays a supporting role in compaction comes from several investigations. Wakasugi (1973)
described a lethal condition in DDK inbred mice that occurred when eggs of these mice were fertilized by sperm from another mouse strain. When fertilized by foreign sperm the survival rate of DDK\(^{+/−}\) zygotes was 5 to 10%. Survival rates of eggs fertilized by DDK sperm (DDK\(^{+/+}\) zygotes), on the other hand, were good and a majority of these zygotes developed to term. Buehr et al. (1987) have further investigated this phenomenon and have shown that DDK mice ova fertilized by C3H/Bi sperm develop altered gap junctional communication. Cells from DDK/C3H/Bi crosses had reduced dye coupling, indicative of poor intercellular communication, that could be corrected by raising the intracellular pH of the embryos. This treatment rescued mutant embryos when applied before spontaneous decompaction, which resulted in cell and embryonic death.

Furthermore, Aghion et al. (1994) have shown that embryos will not compact unless there is gap junctional communication. Okadaic acid was used to inhibit the action of cellular phosphatases which was believed to have caused Cx43 to remain phosphorylated, lowering junctional conductance in cells and resulting in decompaction in these embryos. To further test the importance of gap junctional communication in compaction, Lee et al. (1987) demonstrated that injection of antibodies to Cx43 caused decompaction of cells in an 8-cell mouse embryo. From these experiments it was concluded that gap junction intercellular communication is needed for maintenance of compaction and is necessary for survival of the embryo at this stage.

Expression of Cx43 protein at the 8-cell stage allows the inner cell mass to communicate with the trophoblast cells. The inner cell mass will form the three cell layers
of the embryo proper, while the trophoblast cells implant and form the fetal part of the placenta. After implantation, communication between cells of the embryo and trophoblast becomes more restricted. Cells of the inner cell mass remain ionically coupled to trophoblastic cells, but dye transfer is no longer present (Lo and Gilula, 1979b). This limitation in cellular communication after implantation seems to isolate the inner cell mass from the rest of the developing conceptus, while ionic coupling may allow the inner cell mass to keep pace with outside developments and maintain osmotic homeostasis. Eventual loss of ionic coupling suggests that as development proceeds, the inner cell mass may need further isolation from cellular morphogens in the rest of the conceptus. This progressive isolation of the inner cell mass would allow the embryo proper to follow differential cell development relative to cells of the trophoblast. This barrier to transmission of certain developmental signals may be equally beneficial for tissues outside of the inner cell mass, allowing for uninhibited formation of the embryonic portion of the placenta.

2. Regulative and Mosaic Development

As development continues, temporal and spatial patterns of gap junction expression emerge, beginning within different regions of the inner cell mass and later in the resulting embryo (Ruangvoravat and Lo, 1992; Kalimi and Lo 1989). Transient expression, fluctuation in the level of expression, overlapping regions of specific connexin expression, and the multiplicity of connexin expression in some regions provides the potential for exquisite sensitivity in regulation of intercellular communication. Such regulation may act
as a filter for information available to neighboring cells. For example, two assemblies of cells that communicate with each other may be regulated such that one or the other assembly is effectively isolated from some types of information, but fully affected by other signals. These fluctuations in expression and distribution of gap junctions have been suggested to provide a means by which gap junctions participate in segregation of developmental compartments and establish boundaries in which an organ or other structure can form (Lo and Gilula 1979b).

Two concepts should be noted from the preceding discussion on progressive isolation of the inner cell mass and further compartmentalization of the embryo. First, the amount of intercellular communication provided by the connexon’s channel is regulated as noted by gradual loss of communication in the implanted embryo. There are several means by which this could occur. Reduced communication may reflect a decline in the number of gap junctional channels in the membrane caused by decreased synthesis or increased degradation of Cx43. This would correspond to lowered macroscopic conductance (i.e., cumulative capacity for cell-cell communication mediated by all open gap junctional channels at cell-cell interface). Alternatively, a decline in microscopic (single channel) conductance also could explain diminished communication. This could result either from reduction in the channel size or open time, possibly due to phosphorylation, or could reflect expression of a different connexin.

With regard to the heart there is evidence that supports some of these alternatives. For example, Cx43 channels are gated by phosphorylation/dephosphorylation-driven
events (Godwin et al., 1993). Also, there are at least two other gap junction proteins, Cx45 and Cx40, produced during heart development (Kanter et al., 1992; Reaume et al., 1994; Veenstra et al., 1992). Unitary conductances of Cx45, Cx43, and Cx40 channels differ greatly from one another with maximum conductances of 30ps, 60ps, and 160ps respectively (Veenstra et al., 1994; Moreno et al., 1994; Beblo and Veenstra, 1997).

Second, available information suggests the possibility that the mammalian embryo shifts from a regulative type of development to that of a mosaic type. Regulative development is described by Allen (1987) as the ability to compensate for loss of cells or information. As shown by work of Lee et al. (1987), cells of an embryo that were injected with an antibody to Cx43 were excluded from further development; however, the embryo formed a normal mouse. Thus, remaining cells of the embryo "regulate" and restore missing elements. Even later in development compensation for small losses can take place, but this ability anatomically corresponds to domains of cells that have maintained intercellular communication. For example, at the 8-cell stage all cells are in communication with one another, thus restoration for loss of a few cells is possible. Later, as communication is more localized, ability for restoration is confined to a communication compartment. If most or all of a compartment is lost, there is little to no compensation. Regulative development is therefore dependent on communication between cells both locally and globally.

Furthermore, as the embryo breaks up into smaller and smaller domains, development takes on characteristics of what is described as mosaic development: that is,
each set of cells is destined to become a particular structure. These sets of cells can be described as communication compartments in which all cells are linked by gap junctions and work together to form a particular organ or structure. An example of this type of partitioning of embryonic tissues into developmental fields is known to take place in segmented insects such as *Oncopeltus* and *Calliphora* (Locke, 1959; Lawrence, 1973). However, there is evidence for electrical coupling across boundaries of insect developmental fields (Warner and Lawrence, 1973), but dye exchange is restricted except within cells of the same segment. Mosaic development is most dependent, therefore, on communication between cells within a compartment. Thus, a role for the overlapping regions of specific connexin expression and electrical coupling between compartments in this type of development could be a setting for the presence of a global gradient providing spatial resolution for organs and patterning of the embryo.

3. Egg Cylinder Stage

At about 7.5 days post conception (dpc), as shown in Fig. 4, the murine embryo is at the stage of the egg cylinder. The endometrium in which the embryo implants responds by becoming swollen, and invading cells form a syncytium around the embryo. These cells are less compact than resident endometrial tissue, and in appearance are a thin, loose, areolar layer of cellular bodies that have a firm attachment to the developing egg cylinder only at the polar region. Surrounding this syncytium and encompassing the entire embryo is a thick, more compact, areolar layer of endometrium that has undergone decidual reaction which gives the mouse uterus a beaded appearance where embryos are present.
Figure 4. At 7.5 dpc the embryo is at the egg-cylinder stage and is surrounded by endometrium that has undergone the decidual response. Only the cells that constitute the polar region (PR) have formed trophoblast giant cells, eventually becoming the embryonic component of the placenta. At this stage the embryo is cup-shaped and composed of two layers of cells: a superior ectodermal epiblast, and a subadjacent hypoblast. This particular embryo may have started gastrulation, thus having a third layer of cells: the intervening mesoderm. At this magnification and level of dissection, this picture does not reveal the primitive groove that provides the first evidence of an embryonic axis. Further, the embryo proper is not visible within this phase contrast picture; however, the structural beginnings of an embryo are clearly visible.
Only cells that constitute the polar region have formed trophoblast giant cells and will eventually be located between the embryonic and maternal components of the placenta. In Fig. 4, the uterine layers have been removed and the syncytium has followed the endometrial tissues as they were peeled back to reveal the egg cylinder.

At 7.5 dpc the embryo is cup-shaped and composed of three layers of cells: the superior ectodermal epiblast, the intervening mesoderm, and the underlying definitive endoderm, all of which arise from the epiblast. The process by which these cell groups form is called gastrulation and commences with migration of rapidly growing epiblast cells at 6.5 dpc. Prior to this, shortly after implantation, epiblast cells organize into a simple epithelium surrounding a small proamniotic cavity. The epiblast cells are now polarized, attached at their apices by junctional complexes, and contain subapical concentrations of cytokeratin polypeptides (Jackson et al., 1981). The epiblast is continuous with the extraembryonic ectoderm being tightly opposed to it. However, the epiblast cells are a discrete compartment with respect to gap junctional communication (Lo and Gilula, 1979b) and their basal surface lies on a continuous basal lamina separating them from the subjacent primitive endoderm (Leivo et al., 1980). The division rate of these cells is not uniform throughout the gastrulation-stage embryo but is significantly faster in a subpopulation of cells. In rats this subpopulation of rapidly growing cells is localized to the primitive streak (MacAuley et al., 1993), whereas in the mouse different techniques localized this expansive growth to only the anterior portion of the primitive streak (Snow, 1977). The difference between species may be real or an artifact created by different
techniques employed. Whatever the case, the primitive groove provides the first
evidence of an embryonic axis in the mouse, and the primitive pit the two adjacent surface
depressions furnishes an entry point for the migrating cells between the ectodermal
epiblast and the subjacent primitive endoderm.

E. Mouse Organ Formation

1. Day Eight of Gestation

The 8 dpc embryo can be identified by four pairs of somites. It is shaped much like
the letter U with the primordia of the central nervous system developing on the dorsal
surface of the embryo. The heart can be seen in Fig. 5 as the two endocardial heart tubes
have started to fuse. The heart has developed from a primordium located superiorly to
that of the head process which, due to growth, has moved to a position just inferior to the
head process and anterior to the anterior intestinal portal. The allantois is clearly visible at
the caudal end of the embryo and is fused with the chorion.

The most prominent structures are the neural folds which are elevated and clearly
show the neural axis. These folds will fuse to form the primitive neural tube which will
subsequently differentiate into the brain and all but the most caudal part of the spinal cord.
At 8.5 dpc the neural groove is visible and both the rostral and caudal neuropores are
open; however, as can be observed in later figures, the neural folds fuse and the rostral
neuropore closes resulting in formation of three primary brain vesicles: prosencephalon or
forebrain, mesencephalon or midbrain, and rhombencephalon or hindbrain. The growth
and development of the brain vesicles are easily followed at different gestational days until
Figure 5. The anterior view of an eight day embryo is on the left, the dorsal view is on the right. The age of the embryo can be determined by the number of somites; this embryo has 4 pairs of somites (S) and thus is classified as 8 dpc. Before dissection as shown in this picture it is shaped much like the letter U, with the primordia of the central nervous system developing on the dorsal surface of the embryo. The cut edges of the membranes (M) are continuous with the egg cylinder. The heart tube (H) is seen as the two myocardial tubes fuse. The heart is located just inferior to the head process and anterior to the heart tube, which is fused with the chorion. The most prominent structures are the neural folds (NF) which are elevated and clearly show the neural axis (NA). These folds will fuse to form the primitive neural tube, subsequently differentiating into the brain and reportedly all but the most caudal part of the spinal cord. The somites formed within the paraxial mesoderm subsequently divide into the sclerotome from which the vertebral column is derived, and the dermomyotome from which skin and muscle are derived.
the developing cranium obscures the process by forming in the mesenchyme around the developing brain. Unequal growth in the hindbrain between the midbrain flexure and cervical flexure results in formation of the pontine flexure that causes a thinning of the roof of the hindbrain that is quite apparent at 10.5 dpc. Unfortunately, the differentiation and migration of the cellular components of the brain are obscured from casual observation; this may be the sequence of events most sensitive to cell-cell communication provided by gap junctions.

The somites formed in the paraxial mesoderm (Fig. 5) subsequently divide into the sclerotome from which the vertebral column is derived, and the dermomyotome from which skin and muscle are derived. The body of each vertebra is composed of the caudal part of one somite and the cranial portion of the next somite down the column. Cells of the sclerotome destined to form the vertebra migrate ventromedially, surrounding the notochord. Segments of the notochord not surrounded in this manner expand to form the nucleus pulposusa, the gelatinous center of the intervertebral disc.

2. Embryonic Turning

Prior to 8 dpc the embryo is not covered by the complete set of extra-embryonic membranes resulting from its formation on the outer surface of the egg cylinder surrounding the pro-amniotic cavity. Between 8 and 9 dpc the embryo goes through major changes in size and organogenesis is greatly accelerated. The embryo rotates so that instead of the anterior surface being open and on the outside of the egg cylinder, this surface closes and is relocated to the pro-amniotic cavity which is progressively becoming
the amnionic cavity. This "turning" process marks the point at which the mouse embryo adopts the familiar fetal position seen in other mammalian species.

Simultaneously, the embryo rolls into its extra-embryonic membranes and becomes completely surrounded by its amnion and yolk sac.

The turning event is captured by freeze frame in Fig. 6. The rolling of the embryo into its extra-embryonic membranes is noticed by looking at the direction the tail is pointing (to the back) in the lower left hand portion of the picture and comparing this to the position of the head process (pointing up) in the upper right hand corner of the picture. This embryo has a curve in the "spine" about two thirds down the cranio-caudal axis that makes the embryo look as if someone twisted the animal around to make the anterior portion of the animal visible. This is not an artifact of the picture but the result of the embryo going through this stage of development. A line drawn on the dorsal surface of the embryo would have a spiral shape. The line on the dorsal surface of the 8 dpc embryo would have a U shape; on the dorsal surface of 9.5 dpc embryo it would have the characteristic C shape of a fetal position. Another difference is that the dorsal surface of the embryo is on the inside of the U-shaped curve, whereas the ventral surface of the embryo is on the inside of the C-shaped curve.

Figure 7 provides a diagrammatic representation of the turning of the mouse embryo. The U-shaped embryo starts out the sequence of turning by growing into the amniotic cavity. The movement of the heart primordium from a superior to inferior position relative to the head process pulls the yolk sac and amnion over the embryo. The
Figure 6. Between 8 and 9 dpc the embryo goes through major changes in size and organogenesis is greatly accelerated. This is the time-frame the mouse embryo goes through “turning”. The rolling of the embryo into its extra-embryonic membranes is noticed by looking at the direction the tail is pointing (to the back) in the lower left hand portion of the picture and comparing this to the position of the head process (pointing up) in the upper right hand corner of the picture. This embryo has a curve (C) in the “spine” about two thirds down the cranio-caudal axis that makes the embryo look as if someone twisted the animal around to make the anterior portion of the animal visible. The heart (H) has now become a complete tube and bulges onto the surface of the embryo. The removed heart is seen more clearly on the right. Notice also that the rostral neural pore (RNP) has begun to close; its edges are moving toward one another but still are not fused.
Figure 7. This sequence of diagrams (after those found in Beddington, 1983) illustrates the turning event within the mouse embryo between 8 and 9 dpc. When comparing the previous photograph to this diagrammatical representation, one can see the caudal portion of the embryo flips around as a natural part of the turning process. When the animal was linearized during dissection and photographed in Fig. 6, the rotation of the caudal portion caused a noted curvature in the spine. This diagram also demonstrates how the embryo rolls into the extraembryonic membranes, eventually surrounding the fetus with several protective coverings. Notice how the fusion of the anterior surface continues over time to embody the embryonic endoderm (green) while the embryo continues to grow into the amniotic cavity (AC). This process covers the heart and removes it from the surface of the embryo as seen in the previous photo. All cavities other than the amniotic cavity (AC) become virtual by 10.5 dpc.
Diagram Illustrating the Turning of the Mouse Embryo

- Embryonic Endoderm
- Embryonic Ectoderm and Mesoderm
- Ectoplacental Cone and Trophectoderm
- Reichert’s Membrane
- Extraembryonic (Parietal) Endoderm
- Allantois
- Extraembryonic Mesodermal and (Visceral) Endodermal Components of Yolk Sac
- Mesodermal and
- Ectodermal Components of Amnion
- EC - Exocoelomic Cavity
- AC - Amniotic Cavity
- YC - Yolk Sac Cavity
embryo then begins to roll the caudal portion of its body, from a superior perspective, counterclockwise in preparation for the change in position while at the same time the ends of the embryo are moving closer to one another. Simultaneously the anterior surface is closing and pulling the embryo up into the amnionic cavity, resulting in the extra-embryonic membranes being pulled down over the embryo and closer to one another on the anterior surface of the embryo. Next, the cranial portion of the embryo passes anteriorly to the caudal portion (Fig. 6 pictures the embryo straightened by dissection). As the two ends of the embryo pass one another, the roll is completed and the embryo is in a fetal position.

3. Days Nine Through Sixteen

By 9.5 dpc the embryo has completed the process of turning and has assumed a full fetal position (Fig. 8). Three pharyngeal arches are easily identified in the cephalic region. The mesenchyme of the septum transversum is first seen at this stage; within it the hepatic diverticulum /biliary primordia start to form. Sinusoids, primarily from the viteline venous system, proliferate and soon the liver is the largest organ and takes over production of red blood cells. Small limb buds can be seen developing in the eventual area of the forelimbs, although at this stage the size of the head process relative to the rest of the body makes it appear that these buds could be the hindlimbs.

By 10.5 dpc the embryo takes on a more mouse-like body shape (Fig. 9). The head is more defined with an eye placode formed, giving better perspective to the body plan being developed. The anterior neural tube has been in the process of forming the forebrain
Figure 8. This profile of a 9.5 dpc embryo shows that it has completed the process of turning and has assumed a full fetal position. Three pharyngeal arches (A) are identified in the cephalic region. The heart (H), pictured above, has undergone extensive growth and is no longer a simple tube but is looped with dilations. While still attached to the embryo it can clearly be seen located on the surface of the developing chest. This is the last day that the heart will be visible without removing enclosing sheets of cells. Internalization of the heart has begun, and the septum transversum has started to form. The heart is contracting regularly. Over the next several days its dramatic growth will slow in order to complete fusion and begin the process of remodeling into a four-chambered organ capable of sustaining life outside the womb.
Figure 9. It can be seen that between 9.5 and 10.5 dpc there are dramatic changes in both the body shape and the heart of the embryo. The three segments of the developing brain are now clearly demarcated as forebrain (FB), midbrain (MB), and hindbrain (HB). Facial features are now becoming visible with the development of an eye placode (EP) and the pharyngeal arches are replaced by a mandible. The heart has been internalized and cannot be viewed until removed from the coverings developing over it. The liver (L) is visualized as a very red organ due to the large amounts of blood that are passing through it. Both the forelimbs (FL) and hindlimbs (HL) protrude from the body of the animal; the forelimbs are starting to elongate, and the hindlimbs are little more than paddle shapes. The heart has made dramatic changes in its structure, now exhibiting four distinct sections in its composition indicative of primitive atria (A) and ventricles (V).
and midbrain. Now the hindbrain will begin to go through transitory rhombomeres, an event associated with expression of the family of homeobox genes. The buds for the hindlimbs are now beginning to form as paddles and the forelimbs are starting to elongate. The liver can be observed as a deep red structure about midpoint along the embryo. From 10-12 dpc the lungs, salivary glands and teeth develop in their respective areas in the mouse's body.

By 11.5 dpc the mouse has a complete body plan (Fig. 10). Later development of the gut is the major event that remains to be accomplished and will be completed utilizing borrowed space in the umbilical cord allowing for proper rotation of the gut before it is returned to the abdominal cavity. The primitive midgut expands into the proximal part of the umbilical cord, forming an umbilical hernia. This herniation is present until around 16 dpc when the midgut returns to the abdominal cavity.

The heart has completed its fusion by 8.5 dpc and is a single tube (pictured on the right of Fig. 6). Other changes in circulation include fusion of the extra-embryonic and embryonic vascular systems, which for the first time allow primitive nucleated red blood cells to enter the embryo's circulation. These have developed in the visceral yolk sac and now move into an active role in development of the animal.

Having undergone extensive growth by 9.5 dpc, the heart is no longer a simple tube but a looped tube with two dilatations (pictured at left of Fig. 8). It is clearly seen protruding from the embryo and is at this time contracting with a regular beat. The changes in development of the heart for the next day or so are not so much of growth but
Figure 10. By 11.5 dpc, the fetal mouse has a complete body plan along with a heart that is showing the rudimentary workings of an adult organ. With the fusion of the heart into a single, solid mass, the atria (A) and ventricles (V) are now well-demarcated. From this point the major structural changes occurring are internal relating to the regulation of blood flow and valve formation. External changes involve growth to rapidly expand cardiac output. The different portions of the brain [forebrain (FB), midbrain (MB), and hindbrain (HB)] are expanding and maturing as are the more mouse-like facial features and developing whisker follicles. Limb development is continuing; the forelimbs (FL) appear as if the paddle tissues are preparing for digit formation.
of fusion and remodeling of the blood flow track. After fusion of the two segments of the coiled heart tube, the path of blood flow will play important roles in both structural and functional heart development. The pressure of blood flow through the truncus arteriosus is thought to help in shaping the outlet vasculature of the completed heart and is thus responsible for the final relative positions of the pulmonary trunk and aorta to one another.

The heart is no longer visible as a superficial organ by 10.5 dpc and has four very distinct chambers; the loop of the previous day is now almost completely fused (right of Fig. 9). Serial sections through the heart show the four chambers. Atria can be easily distinguished from the ventricles, which appear to be filled with trabeculae. The endocardial cushion and interventricular septum are being completed, and a thick pericardial membrane encloses all.

Formation of the heart is all but complete by 11.5 dpc. Right and left ventricles are formed and atria are visible. The path of blood flow is controlled by shunts and valves in the heart allowing for bypass of the lungs; the placenta is responsible for oxygen and waste management. During the latter half of gestation, the heart is just growing in size with minor external changes in structure. This developmental progression of the heart can be followed in the series of pictures in Fig. 11; by 16 dpc there is no difference in shape from the adult heart.
Figure 11. The progression of heart development can be followed in this series of images spanning 12.5 to 16.5 dpc where the heart shows very few outward structural changes from that of the adult. At 12.5 dpc the organ has its major components; between 12.5 and 16.5 dpc there is little change in the chambers besides growth. Remodeling during this period is most evident in the great vessels transporting blood to and from the heart.
F. Connexin Expression and Transgenics

1. Expression Patterns

As with limb development, throughout developmental progression of the heart is developmentally regulated multiple connexin gene expression (Wiens et al., 1995). The phosphoprotein Cx45, which may indicate a regulatory mechanism, forms channels with very low conductance and its RNA is developmentally regulated during chick heart development (Laing et al., 1994; Beyer, 1990). There is experimental evidence for developmental changes in regulation of intercellular communication in the chick myocardium (Veenstra, 1991). In this case gap junctional conductance through channels which are not regulated by voltage doubled from days 4-18 of development. This information is consistent with the hypothesis that expression of multiple gap junction genes may confer different regulatory mechanisms on intercellular communication pathways within a tissue. In the human heart, expression patterns of connexins change during cardiac morphogenesis. According to Chen et al. (1994), mRNA of Cx40 was most abundant in fetal hearts and decreased with age, whereas Cx43 and Cx45 mRNA amounts did not vary significantly in the stages examined.

2. Cx43 Knockout Mice

From results obtained from various animal models and discussion in regard to the nature of events needed for compaction and survival of the murine embryo, the mention of delivery (even if by cesarean section) of homozygous knockout mice seems paradoxical at best. A Cx43 null mutant mouse created by gene knockout lacks ability to produce the
Cx43 protein and has raised many new questions about the role of gap junctions in development (Reaume et al., 1994). Although it has been demonstrated that Cx43 is expressed in many tissues and appears to play a critical role in development, knockout mice were originally reported to have had no abnormal morphogenesis in any tissue other than the heart, which is sufficient to cause the demise of the fetus at birth due to anoxia caused by pulmonary stenosis.

When comparing the later gestational heart of a normal mouse to that of a knockout mouse (Reaume et al., 1994) that has lost one of the communication pathways with a different regulatory mechanism, a delay in the developmental progress of the heart in both the hetero- and homozygous pups is noted. Twelve Cx43 knockout embryos were delivered by cesarean section between 17 and 18.5 dpc, and none of the hearts examined were comparable to that of even a normal 16 dpc embryo but appeared to be most similar to those of a normal 12 to 13 dpc embryo. Other investigators raising Cx43 knockout mice have concurred: there is a 3- to 4-day delay in the maturity of the heart of these animals (C. Lo, personal communication).

Homozygous Cx43 knockout mice characteristically develop pulmonary stenosis, a severe narrowing or closure of the outflow tract, essentially obstructing any oxygenation of blood during respiration (Reaume et al., 1995). These animals survive and develop while the placenta is responsible for respiration but are unable to survive for more than several hours postpartum. However, more recent studies suggest that there are subtle defects in skin and possibly other organs with noticeable edema in the abdomen.
(G. Kidder, personal communication). Further, now that heterozygous animals are commercially available, other investigators have observed random sterility in both genders, decelerated growth, and size reduction in conjunction with the aforementioned developmental delay in the heart (C. Lo, personal communication). When compared to the gross anatomical defects observed in antibody-injected *Xenopus* embryos and decompaction of cells in *Murine* embryo development following injection with antibodies against Cx43, the disparity in these deficiencies begs explanation.

The unexpected outcome of the homozygous knockout mice and differences between genetic and antibody studies may be explained by the level at which disruption of intercellular communication occurred. In the knockout model loss of communication was at the expression level, whereas in the *Xenopus* it was at the protein level. In reconciling these observations, one conclusion is that there may be a genetic feed-back loop that regulates production of the Cx43 protein or any of the connexin proteins. Built into this loop is the ability to compensate for failed production of one connexin protein with production of another or for the ability of a co-expressed connexin protein to neutralize to some extent the effect of not having the uniquely regulated communication provided by a particular connexin protein. Thus, in knockout mice this allows for continued development of the embryo. In the case of *Xenopus*, disruption at the protein level does not trigger the compensation mechanism of this feed-back loop; thus, substitution does not take place and defects are more pronounced (Warner et al., 1984). Evidence supporting this contention comes from two observations: one from patients with visceroatrial
heterotaxia correlated with Cx43 gene mutations (Britz-Cunningham et al., 1995), and another in transgenic mice overexpressing the Cx43 gene (Ewart et al., 1997).

3. Visceroatrial Heterotaxia

Visceroatrial heterotaxia (VAH) is most simply defined as a loss of asymmetrical development in the right and left sides of the body. This syndrome can be nearly asymptomatic or cause severe congenital malformations that are incompatible with life. Six patients requiring heart transplants at Loma Linda University (Loma Linda, CA) were found to have mutations in the cytoplasmic tail of Cx43; five of these mutations were in the terminal serine box that will be discussed later in more detail in relation with regulation of cellular communication provided by this protein (Britz-Cunningham et al., 1995). These patients have in common either polysplenia or asplenia syndrome and pulmonary atresia or stenosis. This is of interest because the compensatory connexin protein that could be substituted for the missing Cx43 protein in the knockout mutant may not have the regulation domain of Cx43. In essence it appears the VAH patients also do not have the regulation domain of Cx43 due to mutations in the coding sequence of the gene. Collectively these data reinforce the notion that each connexin has a unique role in development of the organism.

4. Cx43 Overexpressors

In addition, a strain of transgenic mice developed by Dr. Cecilia Lo at the University of Pennsylvania in Philadelphia constitutively produce Cx43 by having an inserted Cx43 gene, transcription of which is driven by the cytomegalovirus (CMV) promoter. These
mice show several defects relating to development of the heart: enlargement of the right ventricle, evidence of subpulmonary stenosis, enlargement of the atrioventricular conduction system, abnormal deployment of coronary vessels, and a disorganized, spongy appearance of the myocardium of the right ventricle. Examination of fetal heart function using in utero doppler echocardiography revealed arrhythmia and increased outflow velocity consistent with outflow obstruction and conduction system defects. The VAH patients, knockout mice, and overexpressing transgenic mice all have a common thread running through their similar phenotypes: deviation from normally regulated Cx43 communication. The conclusion reached after examining the data is that interference in regulation of cell-cell communication provided by Cx43 has deleterious effects on morphogenesis of the heart.

This interference in each case can be related to the mechanism by which phosphorylation of the cytoplasmic tail of Cx43 regulates information being passed to tissues during heart development. The control over when, and to what cells, information flows has been changed in such a way as to make precise management of this system impossible. In knockout mice the protein is not available and information traffic normally relayed by Cx43 is shunted through other channels that apparently are able to partially compensate, allowing development to continue abnormally. The changes in the DNA sequence found in VAH patients result in a similar disruption of organ formation. Transgenic overexpressors seem to overwhelm the phosphorylation mechanism resulting in a phenotype that is remarkably close to the previous two. When the CMV43 mice are
mated with knockout heterozygous animals, the result is a partial rescue but defects persist (Ewart et al., 1997). These results suggest that regulation of communication through these channels is directly related to the amount of protein available and the actions of protein kinases on these membrane channels.

G. Regulation of Gap Junctional Communication

Regulation of communication properties of gap junctions is an important aspect of the function of these proteins in development and day-to-day life of the organism. Phosphorylation is one mode of regulation that is presumably pivotal in the function of some gap junction proteins; this has been most clearly demonstrated for Cx43 (Godwin et al., 1993; Moreno et al., 1994). This connexin has been reported to migrate as 44 and 46 kD phosphorylated forms on SDS-PAGE gels, suggesting that this protein has several phosphorylation sites and may have a more complex mode of regulation (Crow et al., 1990; Laird et al., 1991; Musil et al., 1990b).

1. Phosphorylation by Kinases

Injection of PKA inhibitor protein (PKI) or PKC inhibitor protein (CKI) decreases cell-cell dye transfer in cells that are well-coupled, suggesting that intercellular communication is maintained by phosphorylation mediated by these enzymes (Godwin et al., 1993). This result is further supported by the finding that after intercellular communication is reduced by injection of alkaline phosphatase, cell-cell communication can be reestablished by injection of cAMP-dependent protein kinase (PKA) or calcium-sensitive, phospholipid-dependent protein kinase C (PKC). When alkaline phosphatase is
not injected prior to injection of PKC, this enzyme reduces intercellular communication.

Taken together, these results indicate that the Cx43 channel's ability to transmit signals is modulated by the phosphorylation state of the protein subunits. Further, this suggests that PKA increases and maintains intercellular communication while the effect of PKC is dependent on the phosphorylation state of Cx43 at the time the enzyme acts.

There are several theories as to how phosphorylation of serine residues within the terminal serine box of Cx43 would cause modulation of signal transduction between two cells (Shah et al., 1994, 1996). The accepted model of the connexon contains six protein subunits that act together to form the membrane channel; thus, each hemichannel has six regulatory domains contained in the carboxyl tails of these proteins (Makowski et al., 1977; Nishi et al., 1991; Hall and Gourdie, 1995). The diameter of a connexon is thought to be approximately 6 nm, and center-to-center distance between two connexons within a gap junction approximately 9 nm. (Fig. 2; Lal et al., 1993). It is believed that the six protein subunits act cooperatively, perhaps mimicking the action of a photographic shutter; thus, when phosphorylation takes place the diameter of the connexon can be increased or decreased. This shutter action would be caused by the conformational change induced by phosphorylation of amino acid residues in the tail region. The size of the pore is modified by which amino acid residues in the tail are phosphorylated and/or by the number of phosphorylated residues. This hypothesis provides for graded signal transmission depending on the number and type of phosphorylation events that have
occurred rather than an on/off view of signal transmission as was previously proposed (Verselis et al., 1986).

Modification of signal transmission relative to this work looked primarily at the roles of PKA, PKCε, PKCδ and their phosphorylation of Cx43. The cytoplasmic tail of Cx43 contains a terminal serine box from residues 360-376 (Asp-Gln-Arg-Pro-Ser-Ser-Arg-Ala-Ser-Ser-Arg-Ala-Ser-Ser-Arg-Pro-Arg) in which are serine residues that have the potential for being substrates for these protein kinases (Shah et al., 1994; Shah et al., 1996) (Fig 1). Phosphorylation of these serines is thought to be key to regulation of cell-cell communication; the interplay of these protein kinases has the ability to modulate this process as noted previously. Combining this with the known high levels of PKA within cells of a developmental system would seem to place the major role of reducing communication on PKC.

2. Protein Kinase C

Protein kinase C contains three subfamilies: conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) (Steinberg et al., 1995). All isoforms in this family of protein kinases consist of four conserved domains separated by variable sequences. The C₁ domain is the putative phospholipid binding region and contains Cystine-rich sequences which bind two molecules of Zn²⁺ that are responsible for diacylglycerol (DAG)/phorbol ester activation. C₂ is the accepted calcium-binding domain, leaving two highly conserved carboxyl terminal catalytic domains (C₃ and C₄) which include an ATP-binding site. The isoforms of PKC are grouped by their activation requirements. Activation of cPKC family
members (α, βI, βII, and γ) are dependent on availability of calcium, phospholipids, and DAG or phorbol ester (Steinberg et al., 1995; Ono et al., 1987). The nPKC subfamily (δ, ε, η, and θ) does not have the calcium binding domain and thus activation is not dependent on the presence of this ion (Koide et al., 1992; Chang et al., 1993). The αPKC isoforms (ζ, λ, τ, and μ) require only phospholipids for activation (Ono et al., 1989; Akimoto et al., 1994; Selbie et al., 1993; Johannes et al., 1994). Because differences between the subfamilies are based on requirements for activation and are reflected in the presence or absence of sequences located in their amino-terminal regulatory regions, it may be concluded that the consensus sequence for phosphorylation would not vary between the subfamilies. This is not the case, however; experimental evidence shows that PKC isoforms differ intrinsically in their in vitro substrate specificity (Schaap et al., 1990; Pears et al., 1990; Pears and Parker, 1991; Stabel and Parker, 1991; Schaap et al., 1989; Hug and Sarre, 1993). For all isoforms of PKC the consensus phosphorylation site is best described as a short stretch of basic residues, preferably arginine, followed by the phosphorylatable serine or threonine residue. However, this does not guarantee that every isoform of PKC will act with the same affinity at these sites.

Members of the PKC family are thought to play crucial roles in a wide variety of physiological processes (Sposi et al., 1989; Nishizuka, 1986). Of importance to this study is their role in cellular differentiation and how they influence membrane protein function. Numerous hormones, growth factors and neurotransmitters acting through their membrane receptors stimulate phosphodiesterase hydrolysis of phosphatidylinositol 4,5-
bisphosphate to create inositol 1,4,5-trisphosphate and DAG to activate different members of the PKC subfamilies.

In 1994, Rybin and Steinberg reported results of a study on PKC isoform expression in the developing rat heart. This investigation was important as very few studies had been done to detect which isoforms were expressed in the fetal heart or tissue. These investigators reported that PKCβ was not detected in the heart, while PKCα and PKCζ expression seemed to be limited to non-myocyte cells of the heart; PKCδ was most prevalent in fetal tissues when compared to other time points, beginning with 14 dpc. Overall, PKCε was the most highly expressed isoform of the PKC family in the heart.

There is a paucity of studies relating to the levels and activities of protein kinases in the developing heart; the majority of studies that have been done start at 14 dpc of embryonic rat development. This is long after the major structural features of the heart have been established. Studies during 7-9 dpc, when looping and formation of the heart are occurring rapidly, have not centered on the major protein kinases. The only report relating protein kinase levels to heart development during this early time frame documented expression of Hek2, a member of the eph receptor-protein tyrosine kinase (R-PYK) gene family, and MSK (myocardial SNF1-like kinase), named for its similarity to the yeast SNF1 gene (Ruiz et al., 1994).

The information available on total PKC content in developing rat heart shows an increase for two to four weeks postnatally. After this time, levels decrease by about 40% to those found in the adult (Girard et al., 1986). In the chick model, activity of cPKC
subfamily members is reported to be higher in the atria; this activity increases until the
day of hatching, then declines to adult levels (Wrenn et al., 1988). These authors also
reported that PKA activity in the chick heart was relatively constant until hatching; then
activity decreased until reaching lower adult levels.

3. Cyclic-Amp-Dependent Protein Kinase

PKA resides in the cell as a holoenzyme and is composed of two catalytic subunits
bound to homo- or heterodimers of regulatory subunits (Oyen et al., 1988). Activation of
this protein kinase occurs when two molecules of cAMP bind to each subunit of the
regulatory dimer making the catalytic regions available. There are two isoforms of the
regulatory subunit, each having two types, for a total of four subunits (RIα, RIβ, RIIα,
and RIIβ) available for dimerization and binding to the three different types of catalytic
subunits (Cα, Cβ, and Cγ) (Beebe et al., 1990). Increases in cellular concentration of
cAMP results in phosphorylation of serine or threonine in the consensus sequence Arg-
Arg-X-Ser/Thr-Y, where Y is usually a hydrophobic residue. Responsibility for this
phosphorylation is usually not reported as being divided among various types of PKA but
rather is denoted as total PKA activity.

This total activity has been found to be high in embryogenesis relative to the adult in
several animal models. In guinea pigs, the ratio between PKG (cGMP-dependent protein
kinase) and PKA has been shown to decrease in the lungs and heart as the animal matures
(Kuo, 1975). In the mouse, PKA activity is high at 15 dpc and increases reaching a
maximum at day 7 postpartum (Haddox et al., 1979). The rat model has been the
standard for substantiation of the statement that PKA levels are high in the embryo. Levels for PKA activity possibly as early as 11 dpc in the rat are shown to be high until the first ten days postnatally, with the enzyme being largely in the activated form. However, by day 20 postpartum activity then declines to extremely low levels (Novak et al., 1972). Given these results it seems likely that PKA and PKC activities may well be important during development.

H. Goals and Objectives of Project

The information presented above and evidence gathered over the past ten years highlight the importance of gap junctional intercellular communication in embryonic development. When evaluated from the view of regulation of gap junction-mediated communication by cyclic nucleotides and phosphorylation, information suggests a mechanism by which different molecular signals could direct the growth of an organism. Particularly, because the participation of Cx43 and its regulation by protein kinases has been shown to be meaningful in heart development, it is important to determine how activity levels of the protein kinases expressed in the heart affect the phosphorylation states of this phosphoprotein during the actual structural formation of the organ.

The cell-cell channels formed by this protein are regulated by phosphorylation mediated by PKA and PKC; communication levels between cells could potentially be adjusted by many other enzymes as well. Since studies to date begin to record levels and activities of these protein kinases after structural formation of the heart has been laid and thus no information on activity of PKA and PKC from the beginnings of cardiogenesis
exists, it is essential that these studies be extended to previously unexamined time points. Also, mechanisms by which intercellular communication mediated by Cx43 are regulated in the embryo have not been demonstrated to be identical to those found in the fully differentiated cell or tissues. Therefore, examination of the phosphorylation of Cx43 by PKA and PKC throughout embryonic heart development will give meaningful information relating to interaction of gap junctional communication and structural changes in this organ.

The selection of PKCε and PKCδ for the present study is based on work done by Rybin and Steinberg in 1994. That study centered on isoforms expressed in developing rat heart but dealt with only one time point in the fetal stage (14 dpc), several time points in the neonate, and the adult animal. The conclusion was that, of the subfamilies examined, only protein kinase isoforms α, δ, ζ, and ε were expressed in the fetal heart, and of these only PKCδ and PKCε were substantially expressed in myocytes. Thus, it is these protein kinases that would be acting on Cx43 found in myocardial cells forming the structure of the organ. PKA activity levels are important not only because of the time frames not examined but also because data to this point has relied on histone as a substrate to measure the activity levels of this kinase. More recently developed compounds, such as Kemptide, have proven more effective at measuring PKA activity levels.

Based on this information, combined with unanswered questions left by research completed in the last ten years, this study proposes the following hypothesis: Phosphorylation of Cx43 by PKA, PKCε and PKCδ in embryonic heart cells is identical to
the phosphorylation of Cx43 by these same enzymes in the adult heart; this regulation of communication by phosphorylation corresponds to structural changes in the organ. Thus, as the formation of the heart progresses, the level of expression of each protein kinase is likely to vary in the organ.

In order to test these possibilities, this study will accomplish several goals. First, time points specifically targeting anatomical changes in the developmental sequence of the mouse heart and providing comparative postnatal tissues will be chosen. These time points will start when the heart tube is created by fusion of the two endocardial tubes at gestational day 8.5. Each day from 8.5-16.5 dpc, when the heart has completed anatomical formation, representative hearts will be gathered from litters produced by time-dated pregnancies. Tissues from these developmentally significant times will be used to gather information on Cx43 and the protein kinases of interest, concurrently noting changes that are taking place in the structure of the heart. The later developmental time frame and postnatal tissues will be gathered at 18.5 dpc, 1.5 days post-partem (dpp), 10.5 dpp and from adult animals (approximately 76 dpc).

Second, once these tissues are gathered, the membrane proteins will be isolated from the cytosolic proteins by means of a membrane preparative procedure. The membrane proteins will then be analyzed by Western blot to identify relative amounts of Cx43 at each time point and the different molecular weight species present. Phosphorylated proteins will be identified by comparison with studies completed by other investigators.
Cytosolic proteins will also be analyzed by Western blot for relative amounts of PKA, PKCε and PKCδ at each time point. To further understand how the presence and quantity of these enzymes may interact with Cx43, enzyme activity assays will be performed. Since the two isoforms of PKC are closely related, results of the assays will reflect a combined activity level.

Results from these studies will determine the relative amounts of each of these proteins in the developing heart, revealing whether these parameters change during development of this organ and how they relate to adult protein levels. Data will also show where Cx43 proteins are located, whether these proteins are in a position to function as conduits for intercellular communication, what phosphorylation states of these proteins are, and how these states change during development. Results from in-vitro assays will demonstrate what fraction of each enzyme is active and the levels of expression present in the heart at each time point. Together, these studies will form groundwork for better understanding regulation of Cx43-mediated communication during heart development.
CHAPTER TWO
MATERIALS AND METHODS

A. Timed Gestation

1. Animal Housing

Eight-week-old male and female Swiss-Webster mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana) for the purpose of generating embryos of timed gestation. Female mice were housed in group cages, each cage containing five mice. Males were housed individually to avoid fighting after reaching sexual maturity. Individual cages were separated according to the sex of the animals on shelves in cabinets, each shelf having separate ventilation. The animals were maintained on rodent chow pellets; no supplements such as vitamins or minerals were added to their diet. Water was provided ad libitum and a light/dark cycle of 0600-1800 was used.

2. Animal Mating

Three days before male and female mice were placed together for breeding, they were placed in scent proximity of each other (individual cages were placed on the shelf containing the opposite sex) to induce the ovulation cycle in females. This created a large group of female mice that ovulated in synchrony. A male was then placed with a maximum of three females between 1630 and 1730. The next morning between 0730 and 0830 males were returned to their individual cages and females were checked for a vaginal plug. After 10 to 12 days each female should have ovulated 3 times; if she still had not been found to be pregnant she was again removed from scent proximity for 5 days. The
entire mating process was then repeated with the exception that she was placed singly with a male. If no vaginal plug was found over the 10 to 12 days of this second attempt at breeding and she never showed signs of being pregnant, she was considered physically incapable of reproducing and her tissues were used as controls.

3. Embryo Age Determination

Female impregnation in this species is considered to occur at midnight; therefore zygotes were assigned the gestational age of 0.5 dpc when a vaginal plug was found. Pregnant females were housed together but were removed from the main colony on a separate shelf. This avoided the possibility of delayed implantation or pregnancy termination due to influence of detected scent molecules from a foreign male within the first 24 hours after impregnation. Animals were housed in this manner until embryonic tissues were harvested or the animal was close to giving birth, at which time the female was caged separately for birthing.

There were times when a female was found to have a vaginal plug but was not pregnant. When it was determined that they were not pregnant these animals were returned to the breeding pool before attempting embryo recovery. Determination of pregnancy before harvest of tissues was accomplished by observing abdominal tenting. This can be described as noticing the triangular shape formed by the intersection of two lines tangential to the sides of the animal with their common point of origin at the tail and a third line being drawn horizontally across the chest at the level of the sternum. The more obtuse the triangle, the greater the litter size. In some cases, as with a very small
litter or an obese mouse, this method was not helpful until later in gestation and surgical inspection was the only alternative.

B. Tissue Collection

1. Administration of Anesthetic

All animals were humanely anesthetized prior to extraction of embryos or tissue by an intraperitoneal injection of 0.75 mg/10 g animal weight mixture of butorphanol and sodium pentobarbital (Appendix). A needle was inserted in the middle third of the lower left abdominal quadrant at an approximate 45° angle until penetration of the abdominal musculature was accomplished. Major vascular structures were avoided as injection of anesthetic directly into circulation caused sudden death in some animals.

Animals were placed in a closed container with ether until unconscious and then injected. When ether was unavailable the animal was allowed to grasp the cage top with its forefeet while maintaining the hind legs above the surface of the cage. Use of an insulin syringe with a 26-gauge needle rarely caused enough discomfort to the animal for a struggle to ensue. The injection dose translated into volume to be injected was 0.1 to 0.15 cc per animal on average for earlier gestational time points (8.5-10.5 dpc), but later in gestation 0.2 cc or more was needed depending on the size of the animal. The use of butorphanol to reduce the lethality of sodium pentobarbital and relieve associated respiratory distress that can be observed when sodium pentobarbital is used alone.

2. Dissection Procedures

Once the mouse was unconscious, the skin was lifted from the abdomen with forceps
and a midline incision with scissors was made straight down from this point through both skin and musculature. This opening was enlarged by cutting cranially to just below the ribcage and caudally almost to the vaginal orifice. This incision provided an unobstructed area for tissue removal from the embryos within the uterine horn.

Upon inspection, a non-gravid uterus would lie very close to the spine of the animal and be light pink in color. However, as early as 6 dpc a uterus containing embryos was beaded in appearance and darker in color. Depending on gestational age of the embryos it was possible to pull one uterine horn to the surface of the abdomen and remove the embryos' tissues while the other horn was left within the animal. This helped ensure that tissue was maintained in a state as close as possible to that found within the womb by allowing the mother’s circulation to keep the embryos isolated from effects of tissue collection taking place in the other half of the uterus. However, this technique was not relied upon to prolong working time; tissues have a limited life span (2 hours) after the demise of an animal and thus were removed quickly before the umbilical cord was severed, unless attachment of the embryo to the uterus hindered the process of gathering tissues.

Prior to 11 dpc, the procedure described above was impractical because the embryo was too delicate. Tissue removal required the embryo be extracted from the uterus while submerged in cell culture media or another isotonic solution equilibrated in the cell incubator (37°C). The buoyancy provided by this liquid prevented embryos from becoming indistinguishable from the rest of the conceptus by collapsing into surrounding tissues after loss of amniotic fluid supporting its growth in vivo.
Removal of uterine and extra-embryonic tissues was accomplished in one step for later stage embryos by simply cutting lengthwise down the uterine horn and removing the embryos. When performing the first incision for detaching, 9.5-10.5 dpc embryos sometimes popped out of the enlargements in the uterus marking their site of implantation. However, 8.5 dpc embryos and those embryos that did not liberate themselves from surrounding tissues required a two-step procedure preserving the embryo intact for heart identification and extraction.

After cutting between individual implantation sites and checking to see that the embryo was not ejected because of pressure changes caused by manipulation, musculature of the uterus and surrounding tissues were carefully stripped away and the embryo was placed in clean media. The heart was located and removed immediately. Regardless of age, after extraction hearts were either placed in liquid nitrogen for storage until use, washed in phosphate-buffered saline (PBS) and placed in acetone for immediate processing for use in immunocytological studies, or disaggregated for cell culture.

3. Tissue Storage

When available, hearts were stored in appropriate numbers for use; for example, if Western analysis required ten hearts to be solubilized for protein identification, then tissues at this time point were frozen in aliquots of ten each. Earlier gestational day hearts (before 14 dpc) were transferred with the aid of a pipet to 250 μl microcentrifuge tubes, which were used to hold tissue inside cryotubes. Hearts of sufficient number were centrifuged at 16,000 xg for one minute with a table top centrifuge to compress tissue
sufficiently to remove supernatant medium before storage. Hearts of sufficient size to be
manipulated by forceps were frozen individually.

Western blots were performed after tissues were collected in quantities sufficient for
analysis. Adult tissues were studied first in order to standardize procedures, then more
developed older gestational tissues, progressively using earlier time points of gestation.
This provided experience in handling decreasing tissue sizes and provided information on
quantities of tissue needed to obtain results. By adjusting protocol for maximization of
results while working with decreasing amounts of available tissue per heart, results were
obtained without having to repeatedly collect tissue at these early gestational time points.

C. Western Analysis

Western analysis used sodium dodecyl-sulfate-poly acrylamide gel electrophoresis
(SDS-PAGE) to separate proteins of differing molecular weights (Van Patten et al., 1986;
Fletcher et al., 1986). After transfer of these proteins to a nitrocellulose membrane (Bio-
Rad Laboratories, Hercules, CA), application of antibodies specific for the protein of
interest was used to identify its presence in tissues tested. Adult tissues used to
standardize this technique allowed for consistent results to be obtained using an 8%
stacking gel and a 16% separation gel (30% acrylamide/bis) with an applied electrical field
produced by a Bio-Rad Laboratories model 1000/500 power supply with a constant
amperage setting of 35 mA for gel runs.

1. Gel Run Parameters

The gel electrophoresis chamber was placed within a styrofoam box on a layer of
crushed ice. The volume between the chamber and the box was first filled with packed crushed ice; then ice water was added to a level 3/4 the height of the chamber. This assured efficient transfer of heat from the buffers inside to the ice water surrounding the electrophoresis chamber and a minimization of effects that this heat might have had on migration of proteins through the gel matrix. Instructions for mixing buffers and solutions used for this procedure are listed in the appendix. The ultra-sensitive coomassie brilliant blue procedure was used from time to time to stain gels (Van Patten et al., 1986).

2. Tissue Preparation

Before being allowed to thaw, tissue was removed from storage under liquid nitrogen and placed in detergent-free RIPA buffer within a 1.5 ml microcentrifuge tube. Larger pieces of tissue were mechanically broken up before sonication on ice with a Fisher Scientific 60 sonic dismembrator at a setting of 4-5 watts for 30 sec. as a standard setting or until all the tissue went into solution. The volume of buffer used for sonication depended on gestational age of the tissues; the volume was adjusted in relation to the amount of tissue in order to maintain detectable protein concentrations (Table 1).

3. Cytosolic and Membrane Separation

Tissue suspension was then centrifuged at 60,000 xg with the Beckman Avanti 30 centrifuge for 40 minutes at 4°C. The resulting supernatant containing cytosolic elements was removed and tube walls dried, leaving behind the pelleted membrane fraction to which was added RIPA buffer now containing detergents (Table 1). The membrane fraction was again pulse sonicated at 4 watts to completely solubilize membrane proteins for analysis.
<table>
<thead>
<tr>
<th>Gestational Age of Animal</th>
<th>Number of Hearts Used</th>
<th>Protein Concentration (mg/ml) (M)</th>
<th>Sonication Volume/Protein</th>
<th>Volume/Weight (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>49</td>
<td>2.93</td>
<td>33.4µl/0.070mg</td>
<td>24µl/0.070mg</td>
</tr>
<tr>
<td>9.5</td>
<td>30</td>
<td>3.1</td>
<td>30.5µl/0.070mg</td>
<td>21.9µl/0.070mg</td>
</tr>
<tr>
<td>10.5</td>
<td>18</td>
<td>3.3</td>
<td>28.5µl/0.070mg</td>
<td>20µl/0.070mg</td>
</tr>
<tr>
<td>11.5</td>
<td>13</td>
<td>3.5</td>
<td>34.5µl/0.070mg</td>
<td>20µl/0.070mg</td>
</tr>
<tr>
<td>12.5</td>
<td>8</td>
<td>3.6</td>
<td>7.5µl/0.070mg</td>
<td>7µl/0.070mg</td>
</tr>
<tr>
<td>13.5</td>
<td>7</td>
<td>4.0</td>
<td>10.1µl/0.070mg</td>
<td>7µl/0.070mg</td>
</tr>
<tr>
<td>14.5</td>
<td>7</td>
<td>4.9</td>
<td>20.1µl/0.070mg</td>
<td>6.2µl/0.071mg</td>
</tr>
<tr>
<td>15.5</td>
<td>5</td>
<td>5.9</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>16.5</td>
<td>5</td>
<td>6.0</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>18.5</td>
<td>3</td>
<td>6.9</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>19.5</td>
<td>8</td>
<td>8.9</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>20.5</td>
<td>7</td>
<td>10</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>21.5</td>
<td>2</td>
<td>21.1</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>22.5</td>
<td>1</td>
<td>22.95</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>23.5</td>
<td>1/4</td>
<td>26.54</td>
<td>20.0µl/0.070mg</td>
<td>5.4µl/0.071mg</td>
</tr>
<tr>
<td>Adult</td>
<td>1/8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. This table reflects the internal controls and tissue usage for Western analysis performed on embryonic hearts. As described in the methods section, tissue was fractionated into cytosolic (C) and membrane (M) in the above columns. Amounts of tissue used from the adult heart and liver are estimates; in the actual procedure, these were visualized by filling a microcentrifuge tube to 100µl. Protein concentrations were determined by Bradford analysis and direct UV methods on a Beckman DU-640B spectrophotometer. Sonication volumes listed for the membrane preparations are the first volumes of buffer added to samples before being centrifuged which became the supernatant that was later recovered. Sonication volumes listed for the membrane preparations are the amounts of buffer containing detergents added to the pellet before suspension and loading of the gel.
Whole-cell lysate analysis was accomplished by adding RIPA buffer containing detergents (see appendix for types and concentrations) at the onset of tissue solubilization. These preparations therefore contained elements of both membranes and cell cytosol. Samples were centrifuged at 20,000 xg for 20 minutes at 4°C in order to remove larger particles that did not go into solution.

4. Protein Concentration Determination

Concentration of solubilized proteins was determined by both direct uv280 assay methods (Layne, 1957) and Bradford assay (Bradford, 1976). Materials needed for Bradford assay were provided in kit form (Bio-Rad Laboratories). Concentration of the protein of interest cannot be determined directly by uv280 assay methods unless the protein is purified to make standards of known concentration; however, overall protein concentrations could be estimated. Controls for use in determining protein concentration in experimental samples by both of the above mentioned methods were prepared using RIPA buffer with and without detergents for background readings in respective samples. The standard curve programmed into the Beckman DU640B spectrophotometer was created using bovine serum albumin at known concentrations prepared in solutions identical to those of the heart homogenates. Protein concentrations were determined by data points obtained from the spectrophotometer set to read at a 570 wave length for Bradford assay or a 280 wave length for direct assay (Table 1).

Antibodies used in this study were CT360, anti-protein kinase A, anti-protein kinase Cε, anti-protein kinase Cδ, and MCx40CL. CT360, the generous gift of Dr. Dale Laird,
was raised against the C-terminus of Cx43, amino acid residues 360-382. Generation and characterization of the antibody has been described previously (Laird and Revel, 1990). A 20.7 kD C-terminal protein fragment corresponding to residues 225-406 of the mouse PKA RI subunit was used as an immunogen to create the anti-protein kinase A antibody purchased from Transduction Laboratories, Lexington, KY. A positive control derived from fibroblasts from normal human foreskin accompanied the anti-PKA, Ig2b mouse monoclonal antibody (Tasken et al., 1993; Rohlff et al., 1993; Cho Chung et al., 1990; Taylor et al., 1990). Residues 728-737 of protein kinase Cε and residues 662-673 of protein kinase Cδ were used as immunogens to create the corresponding rabbit poly-clonal antibodies manufactured by and purchased from Calbiochem (La Jolla, CA). All antibodies purchased from research corporations were used in accordance with their instructions. A consensus sequence of the cytoplasmic loop of Cx40 was identified by computer alignment of different reported amino acid sequences found in several animal models. This 22 amino acid peptide, KLRDAEKA KEAHRTGAYEPVA- COOH, was then used by Zymed Synthesis, Inc. (South San Francisco, CA) to raise rabbit antiserum against Cx40; resulting antibodies were thus referred to as MCx40CL. Amounts of this serum to use for Western blotting, including positive (lung tissue) and negative (liver tissue) control samples, were based on ELISA data provided by Zymed.

D. PKA Activity Assay

1. Tissue Preparation

Tissue procured for determination of PKA activity was stored under liquid nitrogen
immediately after removal from the animal. When sufficient amounts of tissue samples (Table 2) were collected, samples were pooled and homogenized in lysis buffer (Appendix). These were centrifuged at 20,000 xg for 20 minutes at 4°C using the Beckman Avanti 30 centrifuge to remove larger particles that did not go into solution. Supernatants were then collected and assayed for enzyme activity at three concentrations: no dilution, 1:2 and 1:5 dilutions.

2. Assay Parameters

Enzyme activity was evaluated with Kemptide as the standard substrate using described procedures (Van Patten et al., 1986; Byus and Fletcher, 1982). Assays were completed under conditions of +/- cAMP, +/- kinase inhibitor (PKI) and +/- Kemptide with positive and negative controls utilizing PKA purified from bovine heart obtained from a slaughterhouse as described (Byus and Fletcher, 1982; Fletcher et al., 1986; Godwin et al., 1993), but with a modified cAMP-affinity resin (Van Patten et al., 1986) being used in the triple column step. PKI was purified by described procedures (Byus and Fletcher, 1982; Fletcher et al., 1986) using a catalytic subunit affinity column as the last step, preparation of which has also been described (Godwin et al., 1993). As a control, the catalytic subunit was inactivated with N-ethylmaleimide (Maller and Krebs, 1977), whereas PKI was inactivated with cyclohexanedione (Byus and Fletcher, 1982).

Twenty microliters of supernatant was added to the reaction tube containing P-32 gamma ATP, total specific activity 3000 μCi, (Amersham Life Science Products, Piscataway, NJ) to bring the volume of the assay to 80 μl. Reaction time was 10 min. for
<table>
<thead>
<tr>
<th>Gestational Age of Animal in dpc</th>
<th>Number of Hearts Used</th>
<th>Sonication Volume (PKA Assay)</th>
<th>Sonication Volume (PKC Assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>100</td>
<td>320 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>9.5</td>
<td>75</td>
<td>320 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>10.5</td>
<td>45</td>
<td>320 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>11.5</td>
<td>30</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>12.5</td>
<td>9</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>13.5</td>
<td>8</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>14.5</td>
<td>7</td>
<td>350 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>15.5</td>
<td>7</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>16.5</td>
<td>7</td>
<td>350 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>18.5</td>
<td>6</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>1.5 dpp</td>
<td>4</td>
<td>400 µl</td>
<td>130 µl</td>
</tr>
<tr>
<td>10.5 dpp</td>
<td>1</td>
<td>400 µl</td>
<td>200 µl</td>
</tr>
<tr>
<td>Adult</td>
<td>1/4</td>
<td>400 µl</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

Table 2. This table reflects the tissue usage for the protein kinase activity assays performed on the embryonic hearts. The amount of tissue used in the assay of the adult heart is an estimate; in the actual procedure the amount was visualized by filling a microcentrifuge tube to 100 µl. Protein concentrations were not obtained by spectrophotometer readings because the resulting tissue homogenate volumes were needed for assays; however, sonication volumes and enzyme concentrations were manipulated to reflect those identified in the Western blotting analysis while maintaining reasonable scintillation counts for accurate determination of specific activities. Sonication volumes listed for the PKA assay preparations are the volumes of buffer added to samples before being sonicated, centrifuged, and recovered from pellet. Sonication volumes for PKC assays, on the other hand, were not centrifuged in an attempt to gain beneficial activation from endemic membrane phospholipids.
all samples at a constant 30°C temperature. Forty microliters of this volume was spotted onto a 2.0 cm diameter circle of Watman P81 filter paper and placed in a 30% acetic acid solution at 4°C to stop the reaction. The assay filters were washed in a 30% acetic acid solution at 4°C, followed by a 30% acetic acid solution at room temperature, and then a 15% acetic acid solution at room temperature for 10 min. each before being rinsed in acetone briefly and removed to dry. After being placed in vials with scintillation fluid, they were counted for one minute on a 3800 Beckman scintillation counter.

E. PKC Epsilon and PKC Delta Activity Assay

1. Tissue Preparation

Hearts used to determine activities of PKCε and PKCδ were stored under liquid nitrogen until sufficient tissue was available to run the assay (Table 2). Tissue collections were pooled and homogenized in activity assay lysis buffer (Appendix). As phospholipids were needed for complete activation of these enzymes, solutions were not centrifuged as were PKA activity assay samples. This assay determined the activity of epsilon and delta isoforms jointly, as they are closely related; an appropriate substrate to distinguish between the two enzymes was not found.

Activity of these protein kinases was determined using a PKCε pseudosubstrate sequence (149-164) Ala159 exchanged for a Ser (Biomol Research Laboratories, Inc., Plymouth, PA), under conditions of previously published protocols (Schaap et al., 1989; Koide et al., 1992; Nakanishi et al., 1993) with a minor modification. Substrate
concentration within reaction solution used in this study was modified to be an average of concentrations used in the above studies. By omission of calcium chloride from assay solutions and addition of EGTA, the reaction solution was created specifically to inhibit action of conventional PKC isozymes that might be present in the tissue.

2. Assay Parameters

The assay was initiated by addition of 20 µl of tissue homogenate to the reaction vial to bring the total volume to 50 µl; the vial was returned to the 30°C water bath. The assay was completed with positive and negative controls using purified PKC from bovine brain. As a positive control, purified enzyme was added to an identical reaction solution with either PKCε substrate or histone (Sigma-Aldrich Corporation, St. Louis, MO) as a pan substrate to test PKCε substrate specificity. To obtain sufficient radioactive counts, the enzyme was allowed to act for one hour; the entire volume was then removed and spotted onto a 2.0 cm diameter circle of Watman P81 filter paper and placed in a 30% acetic acid solution at 4°C to stop the reaction. Filters were processed as described in the PKA assay; after being placed in vials with scintillation fluid, they were counted with the 3800 Beckman scintillation counter.

3. PKC Purification

Purified PKC enzyme used in positive controls was obtained from two bovine brains procured from a slaughterhouse and transported in ice. Both brains were trimmed of meninges, vessels and blood clots while being rinsed in homogenization buffer (Appendix). They were then diced into approximately one inch cubes and homogenized in 1.5 L of
buffer/brain in a commercial blender. The homogenate was then centrifuged at 9000 xg using a JA10 rotor in a Beckman J21 centrifuge for 30 minutes at 4°C. The supernatant was filtered through glass wool. The pH was adjusted to 7.9 with 1M Tris/HCl and conductivity lowered to 0.9 mMho using 12 liters of cold triple-distilled (3-D) water. Purification of the enzyme at 4°C was accomplished by performing the work in a walk-in cold room with all equipment and columns used in the process maintained in this room.

A two-liter packed volume of DEAE-cellulose was removed from storage in .1% benzylkonium chloride /buffer A solution to prevent growth of any organisms. Resin was put through a series of washes, each with a volume of 10 L: 1N NaOH (30 min.), 3-D water (15 min.), 1N HCl (30 min.), and two more washes in 3-D water (15 min. each). Resin was allowed to settle out of solution before each of the above wash solutions were aspirated and discarded. Resin was equilibrated in a five-liter volume of buffer A (30 min.), then pH was adjusted to 7.5 with 1N NaOH (10 min.) while continuously stirring this solution. Two more washes were done in buffer A (30 min), and pH was tested to ensure that it remained stable at 7.5. The DE-52 was then stored in buffer A until use, which should never be longer than a 24-hour period.

The DE-52 ion exchange cellulose that had been equilibrated to a pH of 7.45 and a conductivity of 1.6 mMho the previous day was then mixed with the filtrate and was allowed to batch absorb at 4°C for two hours while being gently stirred. The sedimented DE-52 adopted a yellow appearance indicating that proteins had been removed from solution. After the liquid was aspirated the cellulose was mixed with 1.5 L of buffer A in
order to pour it into a three-liter fritted glass funnel. Once transferred it was washed with an additional 5 L of buffer A. The wash buffer was pulled through and removed from the DE-52 using an applied vacuum leaving damp, packed resin ready to place in the column. The almost-dried cellulose was mixed with another 1.5 L of buffer A and gently poured into a 10.5 x 60 cm column with the outflow open containing one liter of buffer A.

The DE-52 in this column was washed and packed using 3 L of buffer A at a elution flow rate of 10 ml/min., after which it was subjected to a six liter continuous linear 0-0.4M NaCl/ buffer A gradient at a rate identical to that used to wash and pack the column. A total of two hundred sixteen fractions were collected, each containing 28 ml. Every third fraction was assayed for PKC activity; from the results fractions 90-132 were pooled, conductivity was adjusted to 55.8 mMho by adding 100g of NaCl, and the pooled fractions were applied to a phenyl Sepharose column.

Phenyl Sepharose was equilibrated by washing 120 ml of resin twice with 800 ml of 3-D water and twice with 500 ml of 1.5M NaCl in buffer A, then degassing the resin/1.5M NaCl/buffer A mixture overnight. The degassed mixture was then poured into a 2.5 x 30 cm column while preventing air bubble formation or entrapment and packed at 225 ml/hour before the pooled fractions were applied to the column. Enzyme was eluted and collected in fractions of 6 ml each, with a reverse linear gradient of 1.5M to 0M NaCl/buffer A after washing with 2.5 L of 1.5M NaCl/buffer A solution. Enzyme activity was concentrated in the later part of elution, so an additional 450 ml of buffer A was used to remove the enzyme completely.
Conductivity of the fraction pool from the phenyl Sepharose column was adjusted to be identical to that of a 0.1M NaCl/buffer A solution used to equilibrate the protamine agarose column used in the next and final purification step. Equilibration of conductivity is accomplished by addition of buffer A if needed; in our case it was not.

Protamine agarose (Sigma-Aldrich) was packed to a twenty milliliter volume and washed in one liter of 0.1M NaCl/buffer A solution, then initially gravity-loaded onto the column and then packed at a rate of 100 ml/hour. The phenyl sepharose fraction pool was applied to the column at the packing rate, after which the column was washed with 60 ml of 0.1M NaCl/buffer A, followed by 200 ml 0.25M NaCl/buffer A. Elution of the PKC enzyme from the column was accomplished with 500 ml of a 0.25 to 1.5M gradient of NaCl/buffer A collected in 5 ml fractions.

When assayed, fractions showing enzyme activity were pooled and concentrated under nitrogen using an Amicon concentrator with a YM-10 Amicon membrane at 20 psi. Bradford analysis was used to determine enzyme concentration, and a sample of our purified enzyme was run in parallel with purified PKC (provided by Dr. Jolinda Traugh, University of California, Riverside) on a SDS-PAGE-gel stained by ultra-sensitive coomassie brilliant blue procedures to verify the identity and purity of our enzyme.

F. Tissue Culture and Immunocytochemistry

Growth of embryonic heart cells in culture at typical settings used for cell incubation (95% humidity, 5% CO₂ and air, at 37°C) in DMEM containing 10% fetal bovine serum was more consistent when glucose was added to the collection media at a 50mM
concentration. The primary culture of embryonic heart cells was accomplished by removing organs from several animals at one time and placing them on ice in collection media (Dulbecco's modified Eagle's medium - DMEM) containing 10% fetal bovine serum (Gemini Bioproducts, Calbasas, CA) and .02% penicillin/streptomycin (GIBCO BRL, Palo Alto, CA). Early in gestation (8.5-10.5 dpc) thirty or more hearts were needed to get healthy cell colonies.

1. Tissue Digestion

Hearts were then removed from the collection media and rinsed with cold phosphate buffered saline (PBS); organs gathered from more mature embryos (14.5 dpc and older) were minced into fine pieces before being rinsed. The tissue was then placed in a collagenase H (Boehringer Mannheim Corporation, Indianapolis, IN)/PBS solution of 1mg/ml using sterile techniques. Gentle stirring with a magnetic stir bar in approximately 1 ml (depending on the quantity of tissue) of collagenase H solution at room temperature facilitated release of viable cells from the tissue. The collagenase H solution was brought up to 37°C to obtain more rapid digestion and disassociation of cells after the first wash at room temperature. Each cell harvest lasted no more than ten minutes and collection ceased after thirty minutes; cells retrieved after thirty minutes were likely dead or dying.

2. Cell Plating

Cells removed after each digestion were rinsed in PBS and placed in serum containing media within the cell incubator to end the action of any remaining collagenase. All collected cells were pooled and pelleted using a clinical centrifuge set at 500 xg.
Media was removed and cells were resuspended in about 1 ml of fresh incubator-equilibrated media. More or less media was used to resuspend cells the final time before plating depending on the amount of cells harvested. Cells were allowed to adhere to the surface of culture dishes for 24 hours before being washed to remove any cellular debris generated during harvest.

The number of cells per cover slip or culture dish was determined by delivering to each approximately 100 µl of media containing harvested heart cells. When very small amounts of cells were available for harvesting (8.5-10.5 dpc), plating density was increased by allowing the cells to adhere to the substrate area covered by media before adding any additional media to the culture dish.

3. Immunohistochemical Procedures

Methods for detecting Cx43 in cultured cells or heart tissue at 8.5 dpc were identical except PBS was substituted for 0.05% Tween-20 in PBS (TPBS) in preparation of cultured cells. Heart tubes were prepared for immunohistochemistry by the following procedure: organ primordia were removed from embryos (cover slips with adherent cells were removed from media), washed in cold PBS, transferred to cold acetone to remove PBS, fixed in fresh 4°C acetone for 15 min., and washed with TPBS to remove acetone. The specimen was rehydrated in TPBS for 5 minutes before being incubated for 30 minutes in 5% non-fat milk/TPBS solution (w/v blocking buffer) to inhibit nonspecific binding of antibodies. Acetone in conjunction with Tween-20 detergent was used to remove portions of cellular membranes to give access to antibody epitopes for binding.
Negative controls were run in parallel by withholding the primary antibody but using the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich).

CT360 primary antibody was mixed in a ratio of 1:500 with PBS and applied to the tissue for overnight incubation at 4°C after a PBS rinse. The secondary antibody was diluted with PBS per instructions from the manufacturer and incubated with the tissue for three hours in a darkened container.

After being rinsed again in PBS the specimen or coverslips were mounted on slides and viewed with a Zeiss Axiosvert 100 TV microscope with infinity corrected lenses or a Zeiss Universal compound microscope, both equipped with epifluorescence and bright field optics and appropriate filters. Photographic records of results were captured by either a SIT-camera (MTT-SIT, A.G. Heinze Co., Irvine CA) and Image1 software (Analytical Imaging Concepts, Irvine, CA) or a 35 mm camera using Kodak T-Max film with exposure times of two minutes for fluorescence and two seconds for phase contrast, bright field optics. Film was developed in accordance with recommended procedures from the manufacturer.
CHAPTER THREE
RESULTS

Gap junctional communication provided by Cx43 proteins can be regulated in several ways: by increases or decreases in its overall production by mechanisms that regulate gene transcription or protein translation, by modulating the rate by which it is inserted or removed from the membrane of the cell, and by phosphorylation of the protein itself. The main question asked by this study was whether or not anatomical modifications during heart development and maturation could be related to regulatory changes in intercellular communication. If a relationship between regulation of Cx43 and structural remodeling of the heart exists, this would suggest a mechanism by which developmental information relating to organ remodeling during development could be transmitted.

A. Connexin Protein Analysis

Collection of tissue commenced at 8.5 dpc when the earliest observable heart primordia, the heart tube, had formed. Collection continued daily over the time frame of neural crest cell migration and throughout gestation until 16.5 dpc when the heart had completed formation of a four-chambered organ. The hearts were removed from the thoracic cavity of the animals quickly to maintain protein integrity, but care was taken to isolate only tissue that was of cardiac origin.

At each day the organ's external structural changes were noted and Cx43 proteins were analyzed by Western blot methods. Protein content within cellular membranes and changes in phosphorylation states of Cx43 related to changes in gap junctional
communication according to available information on regulation of cell-cell communication. Western analysis of Cx43 over the entire developmental period revealed that embryonic tissues had a different banding pattern than adult tissues.

The several Cx43 species present at different gestational days were identified by assignment of molecular weights to the species based on the corresponding band’s migration in relation to migration of pre-stained molecular weight standards within the same gel. An accurate molecular weight relationship was accomplished by digitizing the gels into the Image1 system (Analytical Imaging Concepts, Irvine, CA), then counting the number of pixels in the vertical distance from each of the two spanning molecular weight standards to the Cx43 protein band of interest; a relative molecular weight based on the distance to molecular weight ratio was then assigned to each Cx43 species. The addition of phosphate atoms to the protein does not significantly increase the measurable molecular weight of the protein, but it does increase separation and aid identification due to conformational changes and additional negative charges that alter the mobility of the protein within the gel matrix.

Over the entire gestational time period described in this study, Western blots revealed five immunoreactive protein bands related to Cx43 (Fig. 12 d). These were assigned the following approximate molecular weights from lowest to highest mobility: 49 kD, 46.5 kD, 44.9 kD, 43.7 kD, and 35 kD. The lowest mobility band occurred primarily at the beginning of gestation, during heart looping, and returned after heart chamber formation. The primary intermediate form (46.5 kD) was present only when
Figure 12. Western analysis of Cx43 was performed using the cellular membrane fraction of cellular homogenates, thus removing cytoplasmic proteins from consideration and focusing only on the proteins that are found within the cell membrane. The profile of the banding pattern for this protein indicates that there are developmental changes within regulation by phosphorylation of this protein throughout development. Heart and liver samples obtained from adult animals were run parallel to all embryonic samples as positive and negative controls. The identification of the sample run in each lane is labeled above the representative lane, all samples being from the heart except those labeled liver, gestational age in dpc until 18.5 then age is in dpp. Molecular weights are denoted on the left of each gel. Membrane (a) is identical to membrane (a) except the molecular weight indicators were removed to label the bands and show the rationale for their identification.
heart tissues were growing rapidly. The secondary intermediate form (44.9 kD) was present primarily in prebirth tissues, whereas the band believed to represent the nonphosphorylated parental form of Cx43 (43.7 kD) was nearly absent until birth. The most mobile form (35 kD) was considered to be a proteolyzed fragment. The intensity of this enzymatically cleaved protein varied during the developmental sequence; perhaps reflecting changes in protein recycling, either within the proteasome after ubiquitination or within lysosomes (Laing et al., 1997; Laing and Beyer, 1995). All Westerns relating to connexin proteins were subjected to membrane isolation protocols; therefore, all proteins identified in these gels were membrane associated.

B. Cyclic-AMP-Dependent Protein Kinase

1. PKA Protein Analysis

PKA protein levels as visualized by Western blot reveal a single band varying only slightly in intensity over the developmental progression (Fig. 13). Changes in the concentration and/or activity of this enzyme in postnatal tissues were not the focus of this study, but our results from postnatal tissues support previous conclusions reported by others, that directly after birth and 10 days post-partem PKA activity is elevated in comparison to adult tissues (Novak et al., 1972).

2. Activity Assay Results

In an attempt to further understand the relevance of these data from the protein analysis to the actual phosphorylating potential available to the cells, protein kinase activity assays were done on supernatants of tissues from every experimental time point.
Figure 13. Protein kinase A Western analysis consistently shows a single band of reactive protein that is identical in position to the positive control (Cont.) that was purchased in conjunction with the antibody. Each lane is labeled with the gestational age (dpc until 18.5 then age is in dpp) of the embryo. Molecular weight indicators are listed to the left of each membrane. There appears to be a peak in protein production at 10.5 dpc; which then seems to drop slightly for several days, followed by an intensification in the signal beginning at 15.5 and continuing until 10.5 dpp. The levels in the adult show a dramatic reduction.
The specific activity data (picomoles of phosphate transferred per millimole of substrate per minute) is demonstrated graphically in Fig. 14. Overall from 9.5 to 30.5 dpc specific activity ratios indicated that the majority of this enzyme was in a fully activated state during heart development (Table 3).

C. Novel Protein Kinase C Family Members

1. PKC Delta Expression

PKCδ was detected as a doublet within embryonic tissues at most time points (Figs. 15 and 16). The identification of two distinct bands and possibly three bands within adult tissue suggests that this enzyme is post-translationally modified. This post-translational modification is likely phosphorylation of the enzyme by the tyrosine kinase c-src or an autophosphorylation of serine643 (Li et al., 1997; Kadotani et al., 1997; Gschwendt et al., 1995). These events have been linked with stimulation of PKCδ activity, its cellular membrane association and shifts in SDS-PAGE gel migration (Gschwendt et al., 1994; Ishikawa, 1996).

2. PKC Epsilon Expression

PKCε was also present at every gestational age analyzed but was barely detectable within the cytoplasm in early embryonic tissues (8.5-9.5 dpc) (Fig. 17a). When analyzed in cytosolic preparations PKCε sometimes appeared as a triplet band (Figs. 17 and 18). The highest mobility band indicates a protein with an approximate molecular weight of 65 kD which conceivably indicates a proteolytic cleavage product when compared to the published molecular weight of approximately 80 kD for PKCε (Schaap, and Parker,
Figure 14. This is a graphical representation of the data acquired from the PKA activity assay. The y-axis on the left is for bar graph representation of the specific activity; the y-axis on the right is for line graph representation of the ratio of PKA fully activated by cAMP within the tissue. Days 11.5, 14.5 dpc and 1.5 dpp of gestation show peaks of activity relative to the days around them, although the peak at 1.5 dpp is much less dramatic in comparison to the others. The activity at 14.5 dpc is unique; not only is the specific activity higher than any other day of gestation, but also the ratio of enzyme activated by cAMP has also peaked. The other two days of increased specific activity are preceded by heightened activated to non-activated enzyme ratios; this ratio declines during the following enhanced activity. The activity ratio for this enzyme is lowest in days 8.5 and 11.5 dpc of gestation and remains low in adulthood. Calculations used in the determination of the specific activity are documented in the appendix. Before birth the age of the animal is in dpc (Days post-conception, 8.5-18.5) and after in dpp (1.5,10.5 Days post-partem). Error bars were generated by using the standard deviation function from Microsoft Excel 7.0 for Windows 95.
<table>
<thead>
<tr>
<th>Gestational Age of Animal</th>
<th>Specific Activity +cAMP/-cAMP</th>
<th>Average Ratio of +cAMP/-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5 dpc</td>
<td>7.42 / 4.53</td>
<td>.440</td>
</tr>
<tr>
<td>9.5 dpc</td>
<td>158.27 / 132.13</td>
<td>.862</td>
</tr>
<tr>
<td>10.5 dpc</td>
<td>181.59 / 176.84</td>
<td>.948</td>
</tr>
<tr>
<td>11.5 dpc</td>
<td>526.56 / 389.40</td>
<td>.668</td>
</tr>
<tr>
<td>12.5 dpc</td>
<td>350.64 / 296.76</td>
<td>.786</td>
</tr>
<tr>
<td>13.5 dpc</td>
<td>337.09 / 311.48</td>
<td>.886</td>
</tr>
<tr>
<td>14.5 dpc</td>
<td>662.70 / 648.40</td>
<td>.957</td>
</tr>
<tr>
<td>15.5 dpc</td>
<td>398.00 / 332.32</td>
<td>.846</td>
</tr>
<tr>
<td>16.5 dpc</td>
<td>345.14 / 325.65</td>
<td>.894</td>
</tr>
<tr>
<td>18.5 dpc</td>
<td>302.28 / 291.97</td>
<td>.973</td>
</tr>
<tr>
<td>1.5 dpp</td>
<td>322.05 / 299.61</td>
<td>.947</td>
</tr>
<tr>
<td>10.5 dpp</td>
<td>195.10 / 174.73</td>
<td>.878</td>
</tr>
<tr>
<td>adult</td>
<td>117.97 / 89.23</td>
<td>.657</td>
</tr>
</tbody>
</table>

Table 3. This table reflects the specific activity data results from PKA assays performed on two replicas of each time point each with a total of ten reactions: five with cAMP activation, and five without. Each reaction was started by the addition of 20 µl of supernatant containing cytoplasmic proteins derived from homogenized heart tissue from embryos at indicated developmental time points. The reaction vial was removed from a water bath set at 30°C, supernatant added, and after vortexing returned to the water bath for a total reaction time of 10 min. Total volume of each assay reaction was 80 µl, half of which was removed, spotted, and processed for counting on a Beckman scintillation counter. For all samples tested enzyme activity was evaluated with Kemptide as the standard substrate using described procedures under conditions of +/- cAMP, +/- kinase inhibitor (PKI), and +/- Kemptide. Positive and negative controls utilized PKA purified from bovine heart. Resulting counts were reduced to background levels by the addition of PKI or the removal of Kemptide from the assay solution, indicating that the counts were specifically from PKA and that endogenous cytoplasmic proteins phosphorylated by other kinases did not contribute significantly to these data points.
Figure 15. PKCδ was detected by Western analysis in both cytosolic and whole-cell preparations. Nitrocellulose membranes were treated with anti-PKCδ antibodies purchased from Calbiochem at a dilution factor of 1:1000. PKCδ was just within detection limits in cytoplasmic preparations at 8.5 dpc but more clearly demonstrated in whole-cell preparations as shown in the single lane to the right of the top image. Western analysis performed on kinases of the same age were from the same tissue sample, thus indicating that PKCδ is present in greater amounts than PKCε at 8.5 dpc. Pre-stained molecular weight standards purchased from Bio Rad are denoted on the left of each membrane; the gestational age of the animal is noted above its corresponding lane in dpc.
Figure 16. This figure is the continuation of the Western analysis of PKCδ again detected in both the cytosolic (a) and whole-cell (b) preparations. Detection methods were identical to those mentioned in the previous figure. Pre-stained molecular weight standards purchased from Bio Rad are denoted on the left of each membrane; the gestational age of the animal (dpc until 18.5 then age is in dpp) is noted above its corresponding lane.
Figure 17. PKCε was detected by Western analysis in both the cytosolic and whole-cell preparations as described in materials and methods. Nitrocellulose membranes were treated with anti-PKCε antibodies purchased from Calbiochem at a dilution factor of 1:1000. PKCε was not detected in the cytosolic fraction at 8.5 days of gestation but could be visualized in whole-cell preparations as noted by the observed protein band in the single lane labeled 8.5 just to the right of the first gel. Pre-stained molecular weight standards purchased from Bio Rad are denoted on the left of each membrane; the gestational age of the animal (dpc) is noted above its corresponding lane.
Figure 18. This figure is the continuation of the Western analysis of PKCε; again, this protein was detected in both the cytosolic and whole-cell preparations as described in materials and methods. Detection methods were identical to those mentioned in the previous figure. These time periods do show the expected triplicate in the whole-cell preparation; however, the corresponding cytosolic fraction analysis did not. It does, however, demonstrate the concentration changes very well. Pre-stained standards molecular weight purchased from Bio Rad are denoted on the left of each membrane; the gestational age of the animal (dpc until 18.5 then age is in dpp) is noted above its corresponding lane.
1990). This conclusion is supported by others reporting similar molecular weight proteolytic fragments (Rybin and Steinberg, 1994) and further by the fact that the concentration of this protein species was greatest during heart development when rapid growth and structural changes occurred which would be expected to coincide with a high degree of proteolysis. In adult tissue samples the concentration of the 65 kD protein species is greatly reduced (Fig. 18 b). The latter two bands suggest a phosphorylated and non-phosphorylated state of the epsilon isoform (Koide et al., 1992; Saito et al., 1992).

3. Combined Assay Results

According to published reports, PKC δ and ε are translocated to the cell membrane upon activation (Eskildsen-Helmond et al., 1997; Bogoyevitch et al., 1993; Ishikawa, 1996; Li et al., 1997; Stempka et al., 1997). PKC δ and ε enzyme activities observed in this study fit well with these published reports on requirements for activation. Detection of enzymes in whole-cell but not cytoplasmic preparations clearly indicate that in early gestation these enzymes were membrane associated. As concentrations within the cytoplasm increased later in gestation there was a concomitant decrease in enzyme activity. Further, since these enzymes appeared to maintain their phosphorylation states while activity was decreasing, it may be that membrane association was a more potent activator of these enzymes.

nPKC family members other than PKC δ and ε are not reported to be major components of the cellular protein array within the heart; also, a substrate was chosen that was reported to be fairly specific to these nPKC family members (Schapp et al., 1989;
Koide et al., 1992). Because of these factors, other members of the PKC superfamily were not considered to have significant influence on recorded enzyme activity (Steinberg et al., 1995).

D. Heart Tube to Loop Fusion (8-11 dpc)

1. Cx43 Expression

During the first four days of development when the heart tube was forming, elongating, and looping (Figs. 6, 8, and 12 a), several different molecular weight species of Cx43 were isolated by Western analysis. However, the species present at 10.5-11.5 dpc were not equivalent to those at 8.5-9.5 dpc; this suggests a change in intercellular communication precipitating the structural changes occurring in the heart tube. During heart tube fusion and elongation but prior to looping (8.5-9.5 dpc), the embryonic Cx43 species consisted of the lowest mobility (49 kD) and secondary intermediate (44.9 kD) forms that make up part of the adult Cx43 complement. The third band, however, represented the primary intermediate form (46.5 kD) that was absent in the adult profile (Fig 12 a). As the tube coiled and began to remodel itself into a single organ with four chambers (10.5 dpc) the quantity of the lowest mobility form of Cx43 (49 kD) was reduced, returning to previous levels the next day (11.5 dpc). The 44.9 kD species was retained, whereas the protein represented by the 46.6 kD band was removed from the membrane concomitant to appearance of the parental Cx43 protein (43.7 kD) during this structural change. When the final steps were taken to complete the fusion of the two segments of the U-shaped organ, reemergence of the 49 kD species and detection
of the parental form of Cx43 at 11.5 dpc may be in response to mechanisms involved in completing the union of these sections.

2. PKA Expression and Activity

During early heart formation (8.5-9.5 dpc) quantities of PKA were detected at similar levels during tube fusion and growth, although specific activity and enzyme activation increased dramatically during elongation (9.5 dpc). Western blot detection methods established that enzyme quantities had a modest increase at 10.5 dpc; this level remained fairly constant over the next 24 hours during loop fusion, but specific activity did not show an elevation until 11.5 dpc (Fig. 13 a). The ratio of enzyme activated by cAMP to that not fully activated is represented by the blue line on the graph in Fig. 14; this shows the ratio was lowest at 8.5 dpc, rose to a developmental peak at 10.5 dpc, then was reduced dramatically to a ratio similar to adult tissue. Over this entire 4-day period, PKA specific activity increased and reached a developmental peak when the heart loop was fusing.

3. PKC Delta and Epsilon Expression and Activity

During the first four time points observed, expression of the nPKC family members revealed that they were not present in equal concentrations within heart tissue. PKCδ appeared to be mostly in the lower mobility form during the first 48 hours of heart development and appeared to be mostly within the cell membrane (Fig. 15 a). This conclusion is supported by the detection of the kinase within the whole-cell preparation containing cellular membranes and the lack of its detection in the cytosolic preparation
where cellular membranes have been removed. Over the next 24 hours (10.5 dpc), observable enzyme expression was diminished to almost undetectable levels. When the heart loop was fusing at 11.5 dpc, the protein signal recovered and nearly matched levels previously detected at 9.5 dpc. The epsilon isozyme of this kinase family increased rapidly over this period from scarcely detectable levels in either the cytoplasm or cell membrane, although more seems to be associated with the membrane (Fig. 17a). By 10.5 dpc enzyme levels were observed to stabilize with little to no amplification in band intensity. Combined activity of PKC ε and δ was greatest in the first two days of heart formation, perhaps reflecting membrane association; by 10.5 dpc the activity had been reduced by half, and by 11.5 dpc the phosphorylating potential of these enzymes was nearly halved again.

E. Chamber Formation and Growth (12-15 dpc)

1. Cx43 Expression

Over the next four days of gestation, when the heart was undergoing chamber formation and growth, Cx43 expression and regulation remained fairly constant. Once again, the 49 kD form was removed from the cellular membrane to be detected only on 15.5 dpc when kinase activity was again modified. The 46.5 kD Cx43 protein band returned at 13.5 dpc and remained throughout this growth period at higher concentrations than were detected previously. The 43.7 kD parental form present at 11.5 dpc was retained for 24 hours but was not detected again until after birth, while the Cx43
regulation state represented by the 44.9 kD protein band was again detected at each gestational day from 12.5-15.5 dpc (Fig. 12 b).

2. PKA Expression and Activity

PKA enzyme levels detected by Western blot methods remained fairly constant with those detected at 11.5 dpc over this developmental period (Fig. 13 b). These static production levels were reflected in all but one of the specific activity data points collected during this four-day period. Specific activity reached another developmental peak on 14.5 dpc, apparently reflecting changes in the activation ratio of PKA rather than any fluctuation in protein level. This ratio, reflecting interaction of PKA with cAMP, increased continually over this time period from that recorded at 11.5 dpc and was then reduced at 15.5 dpc.

3. PKC Delta and Epsilon Expression and Activity

Consistent with results of activity assays were Western blot assays performed on these two isoforms of PKC. The PKCδ banding pattern relating to protein quantity switched from that of the low mobility species being prevalent at 11.5 dpc to that of the high mobility species prevailing at 12.5 dpc, perhaps reflecting decreased phosphorylation or membrane association. Enzyme production then decreased over the next two days until 15.5 dpc when cytosolic concentration dramatically increased (Fig. 15 b). PKCe concentrations remained fairly constant over most of the developmental period from previously detected levels at 11.5 to 14.5 dpc. As with the delta isozyme, the epsilon isozyme's cytoplasmic levels increased at 15.5 dpc, but the increase was not particularly
large (Fig. 17 b). Activity of the PKC δ and ε enzymes increased on days 13.5 and 15.5 of gestation, but these increases were not significant when compared to surrounding data points. Throughout this growth period, specific activity of PKC δ and ε remained at approximately 40% of that detected during the first two days of development.

F. Late Gestation, Birth and Adulthood (16 dpc-1.5 dpp, adult)

1. Cx43 Expression

The 49 kD Cx43 protein band was immediately diminished following its emergence at 15.5 dpc so that at 16.5 dpc it was hardly noticeable (Fig. 12 c). The intensity of this band of Cx43 increased slowly over the latter part of gestation until birth, after which (1.5 dpp) it decreased from prenatal levels (18.5 dpc); this suggests that birth interrupted increasing production of this phosphorylated species of Cx43. Ten days after parturition the more pronounced detection of the 49 kD band again indicated that this species was being manufactured, yet still not at adult levels. The Cx43 species represented by the 44.9 kD protein band was once again present in every time point studied. The embryonic species of Cx43 represented by the 46.5 kD protein band was not present in either the 10-day-old pup or adult tissues but was easily detected prior to these experimental time points. It was apparent by the reemergence of the 43.7 kD band, believed to be representing the parental connexin protein, that phosphorylation of Cx43 in the postnatal tissues was declining as the animal matured.
2. PKA Expression and Activity

PKA may have shown another increase in production at 16.5 dpc when compared to the previous day’s enzyme level, but this new tissue concentration tended to remain static until after parturition (Fig. 13 c). Detected enzyme was diminished in the adult heart when compared with all other time points examined. Not only were quantities of this enzyme apparently reduced, but specific activity remained moderately stable prior to birth (16.5-21.5 dpc) then declined with maturity. The activation ratio was greatest just prior to birth (18.5 dpc) with less dramatic changes immediately before and after this day when compared to the other activity peaks during heart development; following delivery this too was reduced to the lower adult levels.

3. PKC Delta and Epsilon Expression and Activity

In what might be considered the end of heart development after 15.5 dpc, the PKC δ isozyme expression was immediately reduced at 16.5 dpc and remained fairly close to this level until after birth when cytosolic levels were again increased (Fig. 16 a). When estimating PKC δ protein quantity, all embryonic time points were significantly reduced in comparison to adult tissues. These data on the expression of PKC δ were relative to its cytoplasmic component; although phosphorylation of this enzyme is not believed to be a prerequisite for enzyme activation, it may be required for full enzyme activity and its association with the membrane (Ishikawa, 1996; Stempka et al., 1997).

The slight increase in the concentration of the epsilon isoform at 15.5 dpc was reduced in 16.5 dpc tissues (Fig. 18 a). After the decline at 16.5 dpc, increasing quantities
of PKCε were detected in the lower mobility species of the doublet found at earlier
gestational days. Both whole-cell lysate and cytosolic preparations showed protein
concentration increasing from the low levels present at 16.5 dpc to adult levels over the
remaining time points (Fig. 18 b). As with the previous data on the delta isoform of this
enzyme family, the epsilon isotype can also be translocated to the membrane; this
translocation is believed to be related to its level of activity within the cell (Eskildsen-
Helmond et al., 1997; Bogoyevitch et al., 1993). Since these Western analyses were
focused more on the cytoplasmic fraction, they recorded the levels of PKCε that were
likely to be in a less active form.

Western analyses of whole-cell preparations used to identify PKC δ and ε activated
enzyme not observed in cytoplasmic preparations revealed no significant differences
between the two preparations except at 8.5 dpc. The latter part of gestation
(16.5-21.5 dpc) and adulthood were marked by increasing cytosolic enzyme
concentrations as detected by Western blot (Figs. 16 and 18), but the ability of PKC ε and
δ to catalyze the transfer of phosphate declined; by 30.5 dpc it was a third as active as at
16.5 dpc and only an eighth as active as at the start of heart formation. Western blot
results of the cytoplasmic fraction and the whole-cell preparations confirmed the results of
the activity assays: As development proceeded, PKC δ and ε enzyme membrane
association and activity decreased.
CHAPTER FOUR
DISCUSSION AND CONCLUSIONS

It is believed that synchronous contraction of the heart is dependent on the presence of intercellular communication provided by gap junctions which unite all myocardial cells. Some investigators have even suggested that Cx43 is the gap junction protein specifically responsible for this function (Beyer, 1993; Page, 1992; Severs, 1994). Synchronous contraction of the heart primordia beginning at 8-9 dpc in the mouse model was reported by Beddington (1983), and the first synchronous contractions of rat embryo heart have been reported to occur at 10 dpc (Gross, 1938).

Results from immunohistochemical studies with confocal microscopy demonstrate that in the mouse embryo Cx43 could not be clearly localized to cardiac myocytes before 9.5 dpc (Yancey et al., 1992). Several observations from the rat model using immunohistochemical procedures, Northern blot analysis, and Western blot analysis have demonstrated the presence of Cx43 by 13 dpc (van Kempen et al., 1991; Fishman et al., 1991b). However, this is several days after synchronous contraction of the heart begins at 10 dpc, suggesting that Cx43 gap junctions may be unnecessary for the initiation and maintenance of the heartbeat.

Our efforts to localize Cx43 with immunohistochemical procedures performed on mouse heart tissue confirmed earlier reports of no clear visualization of Cx43 plaques at 8.5 dpc using these methods, nor were junctional proteins clearly demonstrated between synchronously contracting cells cultured from hearts removed from embryos at 8.5 dpc.
However, the heart primordia and primary cultures of cardiomyocytes from mice at gestational ages of 8.5, 9.5, 12.5, and 15.5 dpc were observed at certain intervals to maintain synchronous waves of contraction down the heart tube and between adjacent cells in culture. Further, application of the gap junction inhibitor heptanol (Valiunas et al., 1997; Watts and Webb, 1996; Li et al., 1996; Tsai et al., 1995) caused a loss of synchronous contraction between adjacent cells in these cultures while removal of the drug allowed synchrony to return. Even so, visual identification of gap junctional plaques could not be obtained, perhaps due to the junctions being small, and linear or circular, as determined electromicroscopically (Skepper and Navaratnam, 1986; Sugi and Hirakow, 1986; Mazet and Cartaud, 1976).

Western blot results from this study confirmed that Cx43 proteins are present in the membrane of day 8.5 hearts (Fig. 12a). Membrane isolation procedures employed allowed for concentration of the gap junction protein and enabled detection at the heart tube stage. The fact that these proteins are in the membrane and that the cells are contracting synchronously suggests that gap junctions are formed and operating as conduits for cell-cell communication. Western blot results taken together with the cessation of synchronous contraction upon heptanol application indicate that Cx43 is present within the early heart tube and that gap junctions formed by this protein are most likely responsible for the ability of cardiomyocytes to act as a unit.

Major changes in embryonic heart anatomy occur between 8.5-9.5 dpc, 9.5-10.5 dpc, and 11.5-12.5 dpc, during which times growth of the organ keeps pace with
the accelerated growth of the embryo. Heart tube formation begins at 8.5 dpc (Fig. 5) by fusion of the two myocardial tubes that arise from the cardiac plate located superior to the head process. Elongation of the tube takes place during the 12-hour period between day 9-9.5 (Fig. 6); during the following 24-hour period the heart undergoes looping (Fig. 8), ultimately giving rise to the atria and ventricles. Between segments of the loop, the tissues fuse and remodel into a single organ (Fig. 9), while between the future chambers valves form that help control increasing blood flow. Formation of the heart is nearly complete by 11.5 dpc (Fig. 10) with final fusion of the loop taking place and further alterations being completed from 12.5-16.5 dpc (Fig. 11). These modifications relate to the final sculpting of the heart so it is able to adapt to a new system of blood distribution when the animal begins respiration. At 15.5-16.5 dpc, the anatomy is set and the heart is growing rapidly in concert with growth of the entire embryo.

A. Connexin43 Regulation

The Western analysis profile strongly suggests that regulation of Cx43 by phosphorylation, and presumably dephosphorylation, marks the beginning of each of these stages. While the heart tube is forming and growing (8.5 dpc), nearly all of the Cx43 protein is phosphorylated to some extent (Fig. 12 a), reflecting the high activity of PKC detected in activity assays and perhaps some involvement of the moderate PKA activity as well (Figs. 12 and 13). During the time the heart is looping (9.5-10.5 dpc), major reductions in PKC activity occur with a concomitant dramatic increase in PKA catalytic activity. Coinciding with these changes, phosphorylated forms of Cx43 begin to reflect
those found within the adult animal. These results show that for almost every change in activity or concentration of PKA and PKC there is a corresponding modification of the Cx43 phosphorylation pattern that coincides with early stages of heart formation.

By the time the heart loop has fused and the heart is making modifications necessary for lung perfusion (12.5-15.5 dpc) (Fig. 11), PKCε and δ have reduced their original activity by about 60% (Fig. 19), while their concentrations are decreased when compared to earlier time points until 15.5 dpc (Fig. 15 b and 18 b). PKA protein levels at this time are plateaued as well (Fig. 13 b). Although activity of this enzyme is reduced from that of 11.5 dpc and remains stable at this relatively lower level over the next two days, the activity ratio continues to increase (Fig. 14). These enzyme-related events are again reflected in the reduced phosphorylation states of Cx43, with the lowest mobility protein (49 kD) being undetectable (Fig. 12 b). Based on these results, it may be surmised that increased intercellular communication facilitates internal metamorphosis of the valvular system during 12.5-15.5 dpc maintaining synchronous contraction but not the unregulated transfer of morphogenic signals beyond areas of reconstruction. Perhaps these changes regulate the apoptotic formation of the valvular foramen between heart chambers and apoptotic mechanisms sculpting other heart structures.

Regulation of morphogenic signals and apoptotic mechanisms may be related to the disappearance and later return of the 49 kD protein species in cellular membranes. Detection of this Cx43 species in membrane preparations reflects its location within the cell membrane. The communication provided by this membrane incorporation is expected
Figure 19. This is a graphical representation of the data acquired from the PKC ε and δ activity assay. PKC is a family of closely related serine-threonine protein kinases that can be classified into two major categories: calcium-sensitive and calcium-insensitive; PKC ε and δ are members of the calcium insensitive category. The y-axis scale on the left, specific activity, is associated with the yellow line; the y-axis scale on the right, percent activity relative to 8.5 dpc activity, is associated with the blue line. These enzymes are most active in the early-gestational time frame, remain fairly constant at a moderate level of activity during the mid-portion of gestation (11.5-16.5 dpc), then decline over the latter part of gestation to low levels of activity during adulthood. Before birth the age of the animal is in dpc (Days post-conception, 8.5-18.5) and after in dpp (1.5,10.5 Days post-parterm). Error bars were generated by using the standard deviation function from Microsoft Excel 7.0 for Windows 95.
to be restricted, as it is believed to be highly phosphorylated due to the activity level of PKC during 8.5-10.5 dpc (Fig 19). This reduced Cx43 communication component is thought to be related to the accelerated growth of the organ while maintaining ionic coupling for contractions. The reduction in the PKC activity and the corresponding loss of this protein from the cell membrane indicates an increase in the Cx43 communication component during 12.5-15.5 dpc (Figs. 13 and 19 b). This may be related to the need for increased control over growth and the ability to transfer apoptotic vesicles in cell groups (Colombo et al., 1995; Green, 1992) as foramen and valves are created.

When the heart's structure is nearly complete (16.5 dpc) and the organ must develop muscle mass to increase cardiac output compensating for growing metabolic requirements of the embryo (16.5-30.5 dpc), each of these enzymes shifts its activity again. Protein kinase activities change, but in opposite directions until birth: nPKC activity is continually reduced while cAMP activation rises, and PKA activity remains elevated when compared to adult activity (Figs. 12 and 13). Concentration of each enzyme is increased from 15.5-16.5 dpc in preparation for this enlargement (Figs. 15 a, 17 a, and 18 c), but enzyme activity is not necessarily increased. Cx43 marks this developmental milestone with the return of the phosphorylated 49 kD protein that was absent at completion of looping (Fig. 12 c), perhaps partially initiating regulation of intercellular communication that will be maintained throughout life.

Birth is a developmentally significant event that stresses the cardiovascular system; this event is marked by changes in all of the proteins evaluated. PKA activation by cAMP
again peaks for the last time at 18.5 dpc (Fig. 14), indicating that one last developmental change is going to take place; the day following birth there may also be a slight increase in PKA concentration (Fig. 13c). PKCε and δ maintain their rising cytosolic protein concentrations in contrast to their ever-declining endogenous activity (Fig. 19), with a sudden increase in cytoplasmic concentration at 30.5 dpc (Figs. 16a and 18a). This could allow a greater fraction of PKC to be available for activation, which may provide a "back-up" mechanism for any cardiac stresses that occur following birth. Finally, declining catalytic activities of both PKA and PKC seem to be reflected in Western analysis of Cx43 showing a transition to the adult band pattern (Fig. 12c).

Immunoblots of Cx43 also show regular faint bands at approximately 35 kD and occasionally at the 80 kD molecular weight levels as has been reported by others (Laird et al., 1991, see Fig. 5). The 35 kD molecular weight band is likely a degradation product of Cx43 (Laird et al., 1991), whereas the 80 kD species is believed to be an aggregated form of the connexin or of its degradation products which have been previously demonstrated (Manjunath and Page, 1985). These bands were not considered in any of the conclusions drawn in this study about regulation of this protein during development.

Results of Western blot analysis demonstrate for the first time there is differential regulation of Cx43 by phosphorylation events in the embryonic heart. Further, these regulation events are correlated to structural changes in the heart, suggesting that information providing for the correct modifications to organ structure are influenced by the presence of appropriately regulated cell-cell communication. Further, the results
(Fig. 12a) suggest that regulation of intercellular communication during development is distinct from that of the adult.

B. Transgenics

Observations of this study suggest an explanation for the lack of severe anatomical malformations other than pulmonary stenosis in Cx43 knockout animals. Data from Western blot profiles suggest that elongation and looping of the heart during normal development occur in an environment where Cx43 is in a highly phosphorylated state (Fig. 12a). This is especially so at 9.5 dpc when PKA activity increases markedly and PKC activity had not yet declined (Figs. 14 and 19). Even without information on the actual conductance properties of Cx43 channels in these states, it may be concluded that the cellular exchange by gap junctional communication is minimized at this developmental stage. This seems congruent with the putative role of gap junctional communication in cellular growth and information available on the action of these enzymes related to intercellular communication (Loewenstein, 1979; Godwin et al., 1993). This reduced-communication environment allows for rapid growth of the cardiac tube and origination of a four-chambered organ. Intercellular communication in knockout mice would also be reduced in the absence of Cx43 where total gap junctional communication is composed of contributions from Cx40 and Cx45. Thus, in knockout mice there are no anatomical malformations relative to chamber number because the loss of an already minimal Cx43 contribution is not significant at this pivotal time.
Signal transduction provided by Cx43 is most likely increased during 10.5-11.5 dpc as evidenced by a rapid decline in PKC activity (Fig. 19), moderate PKA activity (Fig. 14) and concomitant change in Cx43 regulation (Fig. 12a). This communication increase may be in response to required growth control during structural formation resulting from heart loop fusion, programmed cell death, and interaction of neural crest cells. Signal transduction could again be enhanced by increased PKA activity and greatly reduced PKC activity during 12.5-16.5 dpc (Figs. 12 and 13). The Western blot pattern for Cx43 again changes with phosphorylation states, suggesting an increase in cell-cell communication relative to both the 8.5-9.5 dpc stage and the adult pattern. The absence of the lowest mobility and most highly phosphorylated Cx43 protein band from the previous band pattern possibly reflects reduced PKC activity and results in an increase in cell-cell communication provided by Cx43 (Fig. 12b and c). These communication changes may allow for the correct formation of the aorticopulmonary septum within the bulbus cordis and truncus arteriosus, thus creating the aorta and pulmonary trunk. The described malformations in hearts of knockout animals are predictable when the above regulatory and apoptotic mechanisms are included in the developmental equation.

In contrast in Cx43 knockout mice, the above mentioned signal transduction events mediated through Cx43 channels are lost, possibly explaining the grossly enlarged conus region filled with intraventricular septa but normal chamber formation exhibited by these animals. This derangement of normal communication may result in reduced growth control within cells of the conus region and malformations of the aorticopulmonary
septum. While the cellular complement of Cx40 and Cx45 is able to maintain contraction of the heart (which involves the movement of ions), combined gap junctional communication provided by these connexins is not enough to maintain growth control (by morphogen gradients) within this subset of cells.

There is still an unresolved question related to organ size. Since knockout mice lack Cx43 expression, this should result in a lower-than-normal level of cell-cell communication globally, based simply on the fact that there are no Cx43 junctions. As a result, growth of the entire heart should be uncontrolled in relation to this communication component. However, the hearts of these knockout animals are not enlarged, but as noted earlier are small and immature for their gestational age. Examination of the malformations noted in Cx43 overexpressing transgenic mice provide further insight into this dilemma.

Based on published results that PKC can open closed channels for communication and close open channels for communication (Godwin et al., 1993), in Cx43 overexpressors gap junctional communication should be increased. Greater quantities of the protein result in a decreased probability that PKC would phosphorylate Cx43 proteins after they had been acted upon by PKA. The probability that PKC would close the channels opened by PKA is lowered because the maximum number of Cx43 proteins phosphorylated by PKA at any given activation state would be dispersed in a greater number of total Cx43 proteins relative to a normal mouse. This lowered probability would mean that PKC would be phosphorylating more Cx43 channels prior to PKA, opening even more channels and accounting for increased intercellular communication.
As may be expected, increased gap junctional communication within the Cx43 overexpressing animals results in heart abnormalities. The overexpressors exhibit pulmonary stenosis related to hypertrophy of the right ventricle, abnormal disposition of surface coronary vasculature, and enlarged conotruncal regions characterized by a hemicylindrical out-pouching that extends along the atrioventricular groove and includes the region under the pulmonary arterial root (Ewart et al., 1997). In contrast to the normal developmental regulatory pattern for Cx43 described in this study, the presentation of overexpressors suggests uncontrolled morphogenic signaling possibly related to neural-crest cell migration. Due to increased gap junctional communication when compared to both the knockouts and normal animals, these mice might be further expected to have smaller than normal hearts; however, this has not been reported.

This study in conjunction with data from the knockout and overexpressing mice suggests that Cx43 is responsible for localized growth control in the septa and valves related to the foramen that form during heart development. Further, the present results indicate that growth and development in these areas are modulated by phosphorylation and dephosphorylation events regulating the open state of Cx43. This conclusion rests on reports describing the morphology of hearts from Cx43 knockouts and connexin overexpressing animals, both of which have enlarged conotruncal regions but have not been reported to show abnormalities related to the size of the organ in the newborn animal (Ewart et al., 1997; Reaume et al., 1994). Thus the entire pool of data suggests that any misregulation of the phosphorylation mechanisms controlling cell-cell communication
mediated by Cx43 channels may result in heart malformations not directly related to the size of the organ. This conclusion is further supported by the clinical presentations of VAH patients with noted gene defects within the Cx43 coding region.

C. Protein Kinases

Studies relating to protein kinase levels and activity have focused on the latter part of the gestational period beginning at 14 dpc (Novak et al., 1972; Haddox et al., 1979; Rybin et al., 1994). From the images provided of hearts removed from mouse embryos (Figs. 6-11), it is obvious that by this age the heart is a four-chambered organ and further development is centered around enlarging the existing structure. Protein kinase activities regulating Cx43 gap junctions involved in the trafficking of information related to organization of cardiac tissue would be completed, or nearly so by this time.

PKC is a family of closely related serine-threonine protein kinases that can be classified into two major categories: calcium-sensitive and calcium-insensitive. This study focused on the calcium insensitive PKCδ and PKCε isoforms, which are almost uniquely expressed by myocardial cells, and the only calcium insensitive PKC family members reported to be substantially expressed in myocytes (Rybin and Steinberg, 1994). Western blot analysis of cytoplasmic fractions and whole-cell preparations reveal that enzyme production increases over the entire gestation of the mouse. Protein-loading protocols ensured that the fluctuations in PKCδ and PKCε concentration are unrelated to tissue volume and are in fact changes in enzyme concentrations (Table 1).
According to published reports, PKCδ and PKCε are translocated to the membrane upon activation (Eskildsen-Helmond et al., 1997; Bogoyevitch et al., 1993; Ishikawa, 1996; Li et al., 1997; Stempka et al., 1997). This is supported by the difference in the analyses of the whole-cell (membrane containing) and cytoplasmic (membrane excluding) preparations. At 8.5 dpc the presence of both enzyme isoforms is evident in the whole-cell preparation, but the corresponding cytoplasmic preparation has barely detectable levels of these enzymes. The results of the activity assay shows high activation during this early time point, supporting the identification of these enzymes only in the whole-cell preparation reflecting their membrane association (Table 4). Increasing amounts of these enzymes in the cytosolic preparations reflect decreasing membrane association after 9.5 dpc, suggesting that the majority of these enzymes are not in an activated state. Corresponding activity assays performed support this conclusion showing a continual decrease in activity over this same time period (10.5-30.5 dpc) (Fig. 19).

Although PKCε and δ activity was determined together due to similar substrate and activation requirements, Western analyses may demonstrate differences in developmental utilization of these PKC isotypes. Results suggest differences in temporal activities of these two enzymes that are reflected in cytoplasmic concentrations related to each enzyme’s period of inactivity. The PKCδ isoform shows a higher concentration and greater localization in the membrane at 8.5 dpc than does PKCε (Figs. 14 α and 16 α). This suggests that activity recorded in early time points (8.5-9.5 dpc) may be initiated by the delta isoform and then sustained by the epsilon isoform until around 10.5 dpc when
<table>
<thead>
<tr>
<th>Gestational Age of Animal</th>
<th>Combined Specific Activities of PKC ε and δ</th>
<th>Percent Activity Compared to Gestational Age 8.5 dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5 dpc</td>
<td>.04936</td>
<td>100</td>
</tr>
<tr>
<td>9.5 dpc</td>
<td>.04884</td>
<td>98.9</td>
</tr>
<tr>
<td>10.5 dpc</td>
<td>.02492</td>
<td>50.4</td>
</tr>
<tr>
<td>11.5 dpc</td>
<td>.0141</td>
<td>28.6</td>
</tr>
<tr>
<td>12.5 dpc</td>
<td>.01363</td>
<td>36.2</td>
</tr>
<tr>
<td>13.5 dpc</td>
<td>.0179</td>
<td>36.3</td>
</tr>
<tr>
<td>14.5 dpc</td>
<td>.01343</td>
<td>36.8</td>
</tr>
<tr>
<td>15.5 dpc</td>
<td>.01925</td>
<td>39.0</td>
</tr>
<tr>
<td>16.5 dpc</td>
<td>.01515</td>
<td>30.7</td>
</tr>
<tr>
<td>18.5 dpc</td>
<td>.01141</td>
<td>23.1</td>
</tr>
<tr>
<td>1.5 dpp</td>
<td>.008873</td>
<td>18.0</td>
</tr>
<tr>
<td>10.5 dpp</td>
<td>.005819</td>
<td>11.8</td>
</tr>
<tr>
<td>Adult</td>
<td>.003259</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 4. This table reflects the specific activity data results from the PKC assays performed on six replicas of each data point. Activity of PKC delta (δ) and epsilon (ε) were determined using PKCε pseudosubstrate sequence (149-164) Ala to Ser 159 (purchased from Biomol) at a concentration of 50mM. By the omission of calcium chloride and the addition of EGTA, the reaction solution was created specifically to inhibit the action of conventional PKC isozymes that might be present in the tissue. The assay reaction was started by the addition of 20 μl of tissue homogenate to the vial, bringing the reaction volume up to 50 μl. The mixture was vortexed and returned to the water bath set at 30°C; reaction time was then started. The assay was completed with positive and negative controls, using purified PKC from bovine brain. Purified enzyme was added to an identical reaction solution with either histone as a pan substrate (purchased from Sigma) or PKCε substrate, both of which were readily phosphorylated under assay conditions.
total activity is reduced to half that of the previous day. Concentration differences later in
gestation correspond to slight increases in enzyme activity at 13.5 and 15.5 dpc. The delta
isotype shows a decrease in cytoplasmic concentrations between 12.5-13.5 dpc whereas
the epsilon profile remains fairly constant, suggesting that the increased enzyme activity at
13.5 dpc may be a result of PKCδ activation (Figs. 14 b and 16 b). At 15.5 dpc the
epsilon enzyme shows a moderate increase in its whole-cell concentration whereas the
delta enzyme is substantially increased only in the cytoplasm and not in the membrane,
suggesting that any activity increase would be a result of epsilon membrane incorporation.
The later part of gestation (16.5-30.5 dpc) and adulthood are marked by increasing
amounts of both enzymes within the cytoplasmic components reflecting their removal from
membrane association corresponding to the decline in enzyme activity (Figs. 15 and 17).
Thus, these observations suggest that PKCδ and PKCε activation is sequential throughout
gestation with the delta enzyme being activated before the epsilon enzyme.

Sequential activation of these enzymes may suggest that these isozymes have distinct
roles in embryonic development. This is supported by results of others studying delta
isoenzyme activation. Gschwendt et al. (1994) report that PKCδ is phosphorylated by c-src
which increases its apparent molecular weight by 6 kD and stimulates this enzyme’s
activity. The function of c-src is most closely associated to growth control and cellular
differentiation (Brown and Cooper, 1996). Thus, it may be that c-src is influencing cell-
cell transfer of molecular signals by activation of PKCδ during heart development.
Interaction of these two enzymes may result in phosphorylation of serine and tyrosine
residues regulating communication through Cx43 (Loo et al., 1995; Filson et al., 1990; Azarnia et al., 1988). Evidence suggests that this interaction is mediated only by the PKCδ isozyme (Ueffing et al., 1997).

Interaction of these protein kinases with Cx43 and implications for intercellular communication may be observed by comparing concentration trends of phosphorylated species of Cx43 with the trends of protein kinase concentration and activities. For example, the activity and/or cumulative protein concentration of the PKC enzymes match the concentration of 49 kD phosphorylation species closely throughout development, strongly suggesting an association. These data support the hypothesis that the mechanism of regulation by phosphorylation is present within the embryonic heart as it is within the adult, but that this regulation is modified during development corresponding to structural changes in the forming heart.

Overall the relationship of PKA and nPKC activities are essentially reciprocal. PKC is maximally active at 8.5-9.5 dpc, drops precipitously at 10.5 dpc, and continually declines in activity after 15.5 dpc (Fig. 19); concurrently, PKA activity is minimally active at 8.5 dpc, rises to an early maximum at 10.5 dpc, after which activity does not decline until birth at 21.5 dpc (Fig 14). Following birth, enzyme specific activities decline to low adult levels, but even this is at different rates. Protein quantity trends for the different enzymes are in relative agreement over the developmental period but clearly differ in the adult. Thus, during the first three days of heart formation (8.5-11.5 dpc), PKA, PKCδ, and PKCε levels rise and remain stable relative to one another throughout most of
gestation, but in adulthood PKC isozymes remain elevated while PKA quantities are reduced (Figs. 14-18).

D. Summary

This study describes the developmental sequence of the heart from its earliest tubular stage to its transformation into a four-chambered organ. This developmental sequence can be related to differential regulation of Cx43 and for the first time identifies changes in the phosphorylation of this gap junction protein correlated to these structural modifications. Further, the activities of the protein kinases thought to be responsible for this regulation are described and correlations are drawn between the phosphorylation states of Cx43, enzyme activities and structural modifications of the heart. Finally, the observations of this study are applied to two available transgenic mouse models, and explanations are offered for the different malformations characterizing these animals. In the two transgenic models observed, malformations are not related to chamber number or extreme structural defects as might be expected. The results of this study suggests that Cx43 is directly involved with septa and valve formation relating to the malformations described in these animal models, whereas it is indirectly involved in controlling organ size and chamber structure.
APPENDIX
WESTERN SOLUTIONS

RIPA Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM NaH₂PO₄</td>
<td>1.2 mg/ml</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>8.76 mg/ml</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>0.74 mg/ml</td>
</tr>
<tr>
<td>150 mM BME</td>
<td>1.17 mg/ml</td>
</tr>
<tr>
<td>2 mM PMSF</td>
<td>50 µl/ml of 40 mM stock in isopropanol</td>
</tr>
<tr>
<td>5 mM DIFP</td>
<td>2 µl/ml of solution as prepared by Sigma</td>
</tr>
<tr>
<td>10 mM Na₃VO₄</td>
<td>20 µl/ml of 500 mM stock</td>
</tr>
<tr>
<td>100 mM NaF</td>
<td>20 µl/ml of 500 mM stock</td>
</tr>
<tr>
<td>10 µM Leupeptin</td>
<td>10 µl/ml of 1 mM stock</td>
</tr>
<tr>
<td>2.5% SDS</td>
<td>w/v H₂O</td>
</tr>
<tr>
<td>1.5% Triton X-100</td>
<td>v/v</td>
</tr>
</tbody>
</table>

The first three reagents of RIPA buffer are mixed together and stored as RIPA buffer starter in refrigerator. The other reagents are held as stock solutions, some in alcohols because of solubility requirements, others in 3-D water; reagents are added as buffer is needed for procedure. If tissue is stored frozen, then buffer needs to be applied before thawing of tissue.
Acrylamide stock

Acrylamide 29.2 g/100 ml
N’N’-Bis-Methylene-Acrylamide 0.8 g/100 ml

Mix powdered chemicals with 100 ml of 3-D water. Filter through a 0.22 μm vacuum filter after fully dissolved and store at 4°C in a dark biochemical bottle. Under these conditions stock will polymerize with an acceptable gel matrix, but after a month fresh stock should be made.

1.5 M Tris-HCl, pH 8.8

Tris base 18.15 g/100 ml
3-D water 80 ml

Adjust to pH 8.8 with 12N HCl, then bring volume to 100 ml for each unit of Tris base used. Store at 4°C.

0.5 M Tris-HCl, pH 6.8

Tris base 6.05 g/100 ml
3-D water 60 ml

Adjust to pH 6.8 with 12N HCl, then bring volume to 100 ml for each unit of Tris base used. Store at 4°C.
10% SDS

Dissolve 10g SDS in water and bring to 100 ml. Can be stored at room temperature.

2X Laemmli Sample buffer

- 0.5 M Tris-HCl, pH 6.8: 2.0 ml
- Glycerol: 1.6 ml
- 10% SDS: 3.2 ml
- BME: 0.8 ml
- 1% (w/v) bromophenol blue: 0.04 ml

Store at 4°C in 1 ml aliquots.

10X Running Buffer

- Tris base: 15 g/500 ml
- Glycine: 72 g/500 ml
- SDS: 5 g/500 ml

Add 3-D water to chemicals to make a total volume of 500 ml, then store at 4°C.

When used dilute with cold 3-D water.
**Transfer Buffer**

- 25 mM Tris  
  - 3.03 g/L
- 192 mM Glycine  
  - 14.4 g/L
- Methanol  
  - 200 ml/L

Solution is to be pH-ed to between 8.1-8.4 before the addition of the methanol.

**TBS** - Dissolve the 10X TBS concentrate (comes in kit from Bio-Rad) in 3-D water to a total volume of one liter. Working solution is 100 ml to 900 ml.

**TTBS** - Add 350μl Tween-20 to 700 ml 1X TBS solution.

**10 ml of 16% Acrylamide Gel Solution (enough for two mini-gels)**

- Degassed 3-D water  
  - 2.1 ml
- 1.5 M Tris/HCl  
  - 2.5 ml
- 10% SDS  
  - 150 μl
- Acrylamide stock  
  - 5.2 ml
- 10% (w/v) Fresh (NH₄)₂S₂O₈  
  - 50 μl
- TEMED  
  - 5 μl
8% Acrylamide gel 10 ml (enough for four mini-gels)

Degassed 3-D water 4.8 ml
0.5M Tris/HCl 2.5 ml
10% SDS 100 µl
Acrylamide stock 2.6 ml
10% (w/v) Fresh (NH₄)₂S₂O₈ 50 µl
TEMED 5 µl

Color Reagents in Kit

Color Reagent A - Aqueous P-nitroblue tetrazolium chloride in 70%
dimethylformamide with magnesium chloride.

Color Reagent B - 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide.

Color Development Buffer

0.1M Tris pH 9.5 (Tris solid provided in kit with mixing instructions.)
RIPA Buffer for Tissue Homogenization

This buffer and all gel materials are identical to those of a Western blot assay.

Anesthesia Cocktail

Butorphanol 4.2ml/10.4ml of saline
Pentobarbital 4.2ml/10.4ml of saline

Yields 20.8 ml of cocktail that can be stored at room temperature. When making, follow sterile techniques.

Phosphate Buffered Saline

32.2g NaCl
5.51g Na₂HPO₄
0.70g NaH₂PO₄

Chemicals are brought up to a total volume of four liters with 3-D water using sterile techniques, then filtered through a 0.22 micron vacuum bottle top filter and pH brought to 7.2-7.4 with 1N HCl.
Solution One

5% Tween-20
50 mM Tris-HCl pH 7.5
150 mM NaCl
0.1 mM EDTA

Solution Two

5% Tween 20
100 mM Tris-HCl pH 7.5
200 mM NaCl
2M Urea

Solution Three

4% SDS
10% BME
60 mM Tris-HCl pH 6.8
0.01% Bromophenol Blue

All three of the above solutions are made in 3-D water.
PKA ASSAY SOLUTIONS

Assay Temperature: 30°C
Assay Time: 10 min.
Substrate used: Kemptide at 35 mM
Spot for count: 40 μl
Reaction volume: 80 μl
Specific Activity (SA): 200

Lysis Buffer for Tissue Homogenization

10 mM NaH₂PO₄ (pH 6.8)
5 mM EDTA
0.5 mM Isobutyl-Methylxanthine (bmx)
75 mM NaCl
10 mM NaVO₄
10 mM Leupeptin

Amounts of Lysis buffer used at each gestational age are listed in Table 2. Each tissue sample was sonicated on ice until homogenized, at which time it was centrifuged at 20,000 xg and 40 μl of supernatant was added to reaction volume as source of enzyme.
**2X Assay Buffer**

10 ml of 0.5M MES

4.175 ml of 240 mM MgCl₂

50 μl of BME

10.775 ml of 3-D water.

This will make 25 milliliters of buffer that should be stored at 4°C in one milliliter aliquots until use.

**Reaction Solution**

35 mM Kemptide substrate (20 ml of 140 mM stock per reaction)

0.2 mM cold ATP (0.32 ml of 50 mM stock per reaction)

200 cpm γ32, ATP (see calculation below)

2X assay buffer (20 ml per reaction and used to make stocks)

0.1 mM cAMP for +cAMP reactions (8 ml of 1 mM stock per reaction).

The amount of purified cAMP-dependent protein kinase inhibitor protein (PKI) added to appropriate samples was determined by the amount needed to reduce purified PKA enzyme assay counts to background levels. In this case it was 10 μl of a 1:100 dilution of inhibitor protein stock with 98% activity levels. Assay was run +/- Kemptide to illustrate that none of the counts recorded are from endogenous proteins being labeled.
The amount of radioactive ATP to add varies with each assay performed. To calculate the correct volume in µl of a 10 mCi/ml stock to add to the reaction volume, perform the following calculation: \[ SA \times \text{Reaction volume} \times \text{final ATP concentration (mM)} \times 10^3 \] divided by \[ \text{Stock concentration} \times \% \text{ decay} \times 2.2 \times 10^6 \text{ cpm/ci} \]. This works out to be 32 divided by \( \% \text{ decay} \times 22 \) for the parameters listed.

Calculations for picomoles of ATP per millimole of substrate per minute are based on the following formula: \[ \frac{\text{Counts from sample subtract background}}{\text{Counts from straight ATP} \times \text{millimoles of substrate} \times \text{minutes of reaction time}} \]. For these assays, background counts are obtained by adding assay buffer to the reaction vial in place of enzyme sample and treating as if it were a test vial. Straight ATP counts are obtained by addition of assay buffer to the reaction vial substituting for enzyme, spotting 40 µl, letting it dry and not washing it at all before counting.
PKC ASSAY SOLUTIONS

Assay Temperature: 30°C
Assay Time: 60 min.
Substrate used: Protein kinase Cε, substrate 50 mM
Spot for count: 50 µl
Reaction volume: 50 µl
Specific Activity (SA): 200

Lysis Buffer for Tissue Homogenization

10 mM NaH₂PO₄
5 mM EDTA
0.5 mM Isobutyl-Methyloxanthine (mix)
75 mM NaCl
10 mM NaVO₄
10 mM Leupeptin

See Table 2 for amounts added to tissue at different gestational days for sonication. Samples were not centrifuged after homogenization in order for membrane lipids to interact with kinases. Twenty microliters of each sample was added as the source of the enzyme per reaction tube.
Phospholipid Solution

40 ml of 1 mg/ml L-α-Phosphatidylinositol
40 ml of 1 mg/ml Phosphatidyl-L-serine
40 ml of 0.2 mg/ml Diolely-γ-ac glycerol

These volumes are mixed together and nitrogen gas is used to evaporate the chloroform from the lipids, to which is then added 400 ml of 20 mM Tris-HCl. This solution is then sonicated on ice at 2 watts for one minute, cooled for one minute, then sonicated for another minute.

Reaction Solution

20 mM Tris-HCl (1 ml of 1M stock per reaction)
3 mM DTT (.75 ml of 200 mM stock per reaction)
10 mM MgCl₂ (.5 ml of 1M stock per reaction)
0.4 mM EGTA (0.4 ml of 50 μM stock per reaction)
0.2 mM cold ATP (0.2 ml of 50 mM stock per reaction)
200 cpm p32γ ATP (see calculation below)
50 mM Protein kinase Cε, substrate (12.5 ml of 200 mM stock per reaction)
3-D water (p32γ ATP + water volume has to equal 9.65 ml)
Phospholipid mix (5 ml of sonicated solution per reaction)

The amount of radioactive ATP to add varies with each assay performed. To calculate the correct volume in µl of a 10 mCi/ml stock to add to the reaction volume, perform the following calculation: [SA x Reaction volume x final ATP concentration (mM) x 10³] divided by [Stock concentration x % decay x 2.2x10⁶ cpm/ci]. This works out to be 2 divided by [% decay x 22] for the parameters listed.

Calculations for picomoles of ATP per millimole of substrate per minute are based on the following formula: [Counts from sample subtract background] divided by [Counts from straight ATP x millimoles of substrate x minutes of reaction time]. For these assays background counts are obtained by adding assay buffer to the reaction vial in place of enzyme sample and treating as if it were a test vial. Straight ATP counts are obtained by addition of assay buffer to the reaction vial substituting for enzyme, spoting 50 µl, letting it dry, and not washing it at all before counting.
PKC ENZYME PURIFICATION SOLUTIONS

Stock solutions (1 liter each)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
<th>Amount</th>
<th>pH adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2M Tris-HCl</td>
<td>266.42g</td>
<td>pH to 7.5 using 12M HCl</td>
<td></td>
</tr>
<tr>
<td>220mM EDTA</td>
<td>81.88g</td>
<td>pH to 7.5 using 10N NaOH</td>
<td></td>
</tr>
<tr>
<td>220mM EGTA</td>
<td>83.68g</td>
<td>pH to 7.5 using 10N NaOH</td>
<td></td>
</tr>
<tr>
<td>1M Tris base</td>
<td>121.1g</td>
<td>pH not adjusted</td>
<td></td>
</tr>
<tr>
<td>5M NaCl</td>
<td></td>
<td>584.4g/2 liters of buffer A</td>
<td></td>
</tr>
</tbody>
</table>

Buffer A (50 liters)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl</td>
<td>454.54 ml of stock</td>
</tr>
<tr>
<td>2 mM EDTA</td>
<td>454.54 ml of stock</td>
</tr>
<tr>
<td>2 mM EGTA</td>
<td>454.54 ml of stock</td>
</tr>
</tbody>
</table>

pH of entire solution is adjusted to 7.5 using 12N HCl. BME is added to each amount removed before use in protocol, one milliliter of 15 mM BME per liter of buffer being used.
Homogenization Buffer (3.5 liters)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl</td>
<td>45.45 ml of stock</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>227.3 ml of stock</td>
</tr>
<tr>
<td>2 mM EGTA</td>
<td>45.45 ml of stock</td>
</tr>
<tr>
<td>PMSF</td>
<td>172.4 mg/liter</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 mg/liter</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>10 mg/liter</td>
</tr>
<tr>
<td>BME</td>
<td>1 ml of 15 mM/liter</td>
</tr>
</tbody>
</table>

Addition of protease inhibitors and BME is done just before use as they are inactivated over time.
REFERENCES


Tickle, C., A. Crawley, and J. Farrar. 1989. Retinoic acid application to chick wing buds leads to a dose dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. Development 106: 691-705.


